

Handbook of Experimental Pharmacology 204

Sharron H. Francis
Marco Conti
Miles D. Houslay *Editors*

Phosphodiesterases as Drug Targets

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Phosphodiesterases as Drug Targets

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Preface

The cyclic nucleotides, cAMP and cGMP perform ubiquitous signaling roles. In mammals, one or other or both are connected with the regulation of a panoply of key processes that include learning and memory, cell cycle control, differentiation, inflammation, cardiac functioning, smooth muscle relaxation/contraction, and visual signal transduction, to name but a few. As such, there has been much interest over the years in trying to identify, resolve, and comprehend the signaling systems associated with cAMP and cGMP in health and disease and to determine how this knowledge can be translated to generate novel means of therapeutic intervention.

Psychologically, most of us seem to be geared to a greater appreciation of the creation of objects and material rather than their destruction. Invariably, this translates into our collective approach to scientific problems. Certainly, in this regard, the G-protein-coupled receptor (GPCR)-stimulated generation of cAMP has attracted enormous attention over the past three decades. This interest has been translated into effective therapeutics that have exploited the diversity of receptor subtypes and their cell type-specific patterns of expression and been greatly facilitated as their binding site is exposed at the cell surface. More recently, enthusiasm for studying the enzymes that generate cGMP and mediate the cGMP-signaling pathway and its potential for drug targets have emerged. Efforts to target pharmacologically the enzymes that break down cyclic nucleotides, i.e., the cyclic nucleotide phosphodiesterases (PDEs) have more recently materialized, and some of the drugs produced in such programs have proven to be spectacularly successful in the clinic.

For many years, it has been an apparent conundrum that many organisms, including mammals together with lower organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, have stockpiled a mass of PDEs that catalyze the destruction of cAMP and cGMP. Work from many laboratories, including those contributing to this volume, has shed light on why nature has seen fit to not only conserve this diversity but also to elaborate on it throughout evolution. Thus, we see PDEs that have different affinities for cAMP and cGMP, such that low K_m enzymes can scavenge and ensure that signaling systems are truly switched off in resting cells, but then there are sets of PDEs with higher K_m values that “kick in” as cyclases are activated to produce more cAMP or cGMP in cells stimulated by various signals. PDEs also have regulatory features that (a) mediate negative

feedback pathways, which accelerate cyclic nucleotide hydrolysis; (b) provide for selective localization of particular PDE isoforms within a cell so as to confer precise regulatory control upon specific, spatially constrained cellular processes; and (c) provide for cross-talk with other signaling systems so as to integrate cellular responses. Some PDEs are activated through phosphorylation by cyclic nucleotide-regulated protein kinases and so provide a pivotal part of the cellular desensitization mechanism to the corresponding cyclic nucleotides.

Finally, over the last decade, we have seen the advent of genetically encoded sensors for both cAMP and cGMP. These have allowed for the visual appreciation of a phenomenon that has been inferred but, until this time, neither fully proven nor fully accepted, namely that both cAMP- and cGMP-signaling events are compartmentalized in cells. However, for this, you need the targeted, rather than the mass destruction of cyclic nucleotides, for such gradients to form in cells. Tethered subpopulations of cAMP and cGMP sensors subsequently interpret these PDE-shaped gradients. This new understanding offers a pivotal insight into the “why so many PDEs” conundrum. Thus, a large library of PDEs is available where individual isoforms are expressed on a cell type-specific basis. This allows targeting of particular PDEs to specific intracellular sites, membranes, and signaling complexes within cells so as to shape gradients and gate the activation of sensors around them. It is the diversity of PDEs, expressed on a cell type-specific basis with specific functional roles that offers potential for therapeutic exploitation.

The hope for the first PDE therapeutic was aimed at developing selective inhibitors of PDE3 for treatment of heart failure. The first clinical trials were performed with milrinone, which although enhancing cardiac function as hoped, was never mass-marketed as it gave rise to an increase in death rates due to arrhythmias. However, these unfortunate effects were most likely exacerbated by the fact that the patient cohort evaluated were end-stage patients; moreover, milrinone at higher concentrations can inhibit other PDEs. Nevertheless, milrinone is still used under hospital supervision and, furthermore, the highly specific and high affinity PDE3-selective inhibitor, cilostamide is approved for use in intermittent claudication and has no known arrhythmogenic effect.

The concept that PDEs are promising drug targets has been spectacularly extended with selective inhibitors for the cGMP-hydrolysing PDE5. These compounds found a commercial niche for treating penile erectile dysfunction although the first of these compounds had ancestors that originated from programs designed to develop drugs for treatment of heart disease. Since then PDE5-selective inhibitors have progressed to being approved for treatment of pulmonary hypertension and, ironically, may progress back to a new found utility in the treatment of heart disease and other cardiovascular maladies. There has also been a huge effort by the pharmaceutical industry in developing selective inhibitors for members of the PDE4 family. Unfortunately in the race to do this, the generation of a multitude of such compounds ran well-ahead not only of our understanding of both the diversity of isoforms within the four genes PDE4 family, but also ahead of our understanding of their functional roles and structures. Undoubtedly, this has led to a lot of frustration over the years in appreciating which PDE4 isoform is the

“true target” in a particular tissue/cell type and how to deal with adverse side effects of such drugs, such as nausea. Nevertheless, we now have just seen approval for the first PDE4-selective inhibitor, which is being used as a therapeutic to treat chronic obstructive pulmonary disease (COPD). However, recent major advances in our understanding that particular PDE4 isoforms can perform specific functional roles through targeting to signaling complexes, plus new structural insights into how regulatory domains interact with catalytic units bodes well for subsequent generations of PDE4-selective inhibitors. These are likely to address additional therapeutic areas including cognition, psychosis, and cancer. In addition to this, a number of research programs are vigorously pursuing inhibitors of PDE10 for treatment of neuropsychological disorders.

This is then an exciting time for PDE research and development of drugs that target specific enzymes within the myriad of PDEs encoded by the human genome. Each PDE appears to have a specific functional role that affords novel opportunities for development of specific therapeutic interventions. The ability for genetic ablation of particular PDEs, coupled with siRNA-mediated knockdown of specific PDEs and the use of novel dominant negative approaches provide means of comprehending function and further defining potential targets. Furthermore, the huge increase in structural insight of catalytic and regulatory domains of PDEs has transformed our ability to optimize the design of specific inhibitors, and we look forward to the insights that will be derived from the resolution of more complex structures involving not only full-length PDEs, but also for PDEs in complex with specific partner proteins. The ability to assess changes in cAMP and cGMP around specific functional signaling modules will allow not only new biological insights but will also provide the potential for screening for new therapeutics.

Given the limitation in budget, we are inevitably constrained in what we can present. However, in the collection of articles in this volume, we hope to give you a taste of some of the exciting ideas and developments that are currently emerging in this dynamic and important field and how future therapeutic exploitation is currently shaping up. We hope that you enjoy and are inspired by reading them as much as we have been.

Glasgow, UK
San Francisco, USA
Nashville, USA

Miles D. Houslay
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Phosphodiesterase Inhibitors: History of Pharmacology

Christian Schudt, Armin Hatzelmann, Rolf Beume, and Hermann Tenor

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Abstract The first pharmacological investigations of phosphodiesterase (PDE) inhibitors were developed with the clinical efficacies of drugs isolated from coffee, cacao and tea but only later their relevant ingredients were identified as xanthines that act as PDE. With its diuretic, inotropic and bronchodilating clinical efficacy, use of theophylline anticipated the clinical goals, which were later approached with the first-generation of weakly selective PDE inhibitors in the period from 1980 to 1990. Pharmacological and clinical research with these early compounds provided a vast pool of information regarding desired and adverse actions – although most of these new drugs had to be discontinued due to severe adverse effects. The pharmacological models for cardiac, vascular and respiratory indications were analysed for their PDE isoenzyme profiles, and when biochemical and molecular biological approaches expanded our knowledge of the PDE superfamily, the purified isoenzymes that were now available opened the door for more systematic studies of inhibitors and for generation of highly selective isoenzyme-specific drugs. The development of simple screening models and clinically relevant indication models reflecting the growing knowledge about pathomechanisms of disease are summarised here for today’s successful application of highly selective PDE3, PDE4 and PDE5 inhibitors. The interplay of serendipitous discoveries, the establishment of intelligent pharmacological models and the knowledge gain by research results with new substances is reviewed. The broad efficacies of new substances in vitro, the enormous biodiversity of the PDE isoenzyme family and the sophisticated biochemical pharmacology enabled Viagra to be the first success story in the field of PDE inhibitor drug development, but probably more success stories will follow.

Keywords Asthma · COPD · PDE inhibitors · PDE4 inhibitors

1 Introduction

Drug discovery up to 1970 usually happened in small research departments which represented a minor activity of a chemical company. The research team consisted of chemists and pharmacologists. The latter were educated as medical doctors and thus knew the areas of urgent medical need. This team decided about the goals of research (not the board) – mainly on the basis of available resources – and defined

the three essentials (1) the disease indication, (2) the chemical compounds which should be examined or derivatised and (3) the armamentarium of models which were available and could be used for the intended project.

The clinical indications needed to be (1) painful, (2) life-threatening and (3) “ethical”. The pharmacological models had to be designed to deliver read-outs for (1) therapeutical efficacy, (2) dosage and (3) side effects. These parameters were evaluated in anaesthetised intact animals suitable for measuring respiration and cardiovascular and metabolic parameters. Efficacy and potency of various compounds were compared and quantified in isolated organ preparations. These models detected either contractions or relaxations of muscle preparations that had been developed since the beginning of the twentieth century. They provided dose–response curves, were independent from the whole organism and contributed essentially to the selection of new chemical entities (NCEs), which for patent and commercial reasons needed to show “significant advantage and progress” over existing medications.

Most problematic was the source of chemicals used for investigations. The available compound sources were (1) antagonists (of or toward) hormones and mediators, (2) alkaloids which were highly effective but tremendously toxic and (3) some heterocyclic compounds which were used as diuretics, antihypertensives, cardiotonics, pain killers or anti-infectives. Each new project had to be initiated with a long and careful search in the chemical literature for identification of structures which might eventually be active in the biological system of question. The yield, however, was usually low, and even in the middle of 1970, most new developments were called “me-too” compounds developed on the basis of already existing, less effective drugs. After performing toxicologic testing, these substances could eventually be administered to humans. The important tasks of pharmacology were to (1) provide evidence for efficacy and proof of concept *in vivo*, (2) identify a relevant dose in combination with pharmacokinetics and (3) define a safe “first dose in man”. Elucidation of the mechanism of action (MoA) was desirable but not considered to really be necessary. The area of phosphodiesterase (PDE) inhibitor provides several examples for this fast track path to clinical application in the period from 1977 to 1985 (summarised in Table 1).

In the years between 1950 and 1970, basic biochemical research was successful in isolating many enzymes and clarifying their functions, identifying key enzymes of metabolism and starting to analyse molecular mechanisms of diseases. Examples for pioneering research with prominent key enzymes, which were snatched up by pharmaceutical industrial laboratories, were HMG-CoA reductase for cholesterol synthesis, angiotensin-converting enzyme (ACE) for regulation of blood pressure, xanthine oxidase for gout and Na/K-ATPase and the H/K-ATPase for gastric acid secretion. These drug targets were established with considerable success in the years between 1970 and 1980. Enzymes and isolated cells – primary and immortalised – provided a completely new armamentarium for use in pharmacological research. About 10- to 100-fold more measurements per day became possible and identification of true new lead structures from the pool of stored chemicals in each company became a real possibility. Beginning in 1990, highly automated versions

Table 1 Clinical studies with prototypical PDE inhibitors

Inhibitor	PDE	Indication	Assessment	References
Theophylline	Unsel	Asthma, COPD	Symptoms	Hirsch (1922)
Amrinone	3	Chronic heart failure	Myocardial contractility	Benotti et al. (1978)
Enoximone	3	Pulmonary hypertension, COPD	PAH, bronchodilation	Leeman et al. (1987)
Rolipram	4	Depression	Depression scale	Horowski et al. (1985)
Ro 20-1724	4	Psoriasis	Disease score	Stawiski et al. (1979)
Denbufylline	4	Multi-infarct dementia	Psychometry	O'Connolly et al. (1988)
Zaprinast	5	Exercise-induced asthma	FEV1 during exercise	Rudd et al. (1983)
Zardaverine	3/4	Asthma	FEV1	Brunnée et al. (1992)
Papaverine	Unsel	Erectile dysfunction	Penile erection	Brindley (1982)
Benafentrine	3/4	Bronchoconstriction in volunteers	Methacholine-induced FEV1 decrease	Foster et al. (1992)
Tolafentrine	3/4	Pulmonary hypertension	PAH	Ghofrani et al. (2002b)
Sildenafil	5	Erectile dysfunction	Penile erection	Boolell et al. (1996)
		Pulmonary hypertension	PAH, exercise tolerance	Ghofrani et al. (2002a)
Cilostazol	3	Intermittent claudication	Walking distance	Kumar et al. (2007)
Piclamilast	4	Rheumatoid arthritis	Disease score	Chikanza et al. (1996)
Cilomilast	4	COPD	FEV1	Compton et al. (2001)
Roflumilast	4	COPD	FEV1, acute exacerbations	Calverley et al. (2009)

Clinical studies are listed which have been performed with prototypical, advanced and "first-in class" PDE inhibitors. Theophylline and papaverine are denominated as unselective (unsel) PDE inhibitors

of these procedures were established, and high throughput screens testing more than 10,000 compounds per day were the pride of every scientific board. The role of classical pharmacology was now to compare compounds preselected by biochemical methods and to characterise candidates with regard to (1) *in vivo* potency, (2) duration of action, (3) efficacy and (4) adverse effects. In view of the change in the approach of drug development to specific (enzyme) targets, PDE research was a latecomer due to the continuous discovery process of multiple PDE activities and the insufficient insight into relationship between PDE subtypes and pathological functions. However, as the complexities of the PDE superfamily were slowly defined and isolated PDEs became available for study, a greater understanding of their function emerged.

A history of the pharmacology for drugs that inhibit PDE needs to start with the role of the ancient PDE inhibitor theophylline, which was in use therapeutically long before its biochemical action was characterised in 1958. Independently of its biochemical function, theophylline stimulated myriads of pharmacological investigations. Its clinical use followed the pharmacological studies, and despite being

accompanied by substantial adverse effects (AEs), its use anticipated efficacy in several diseases which are currently treated by selective PDE inhibitors or will be in the near future. After identification of PDEs and theophylline as a PDE inhibitor in 1958, it took nearly 20 years before the clinical potential of new PDE inhibitors was recognised. Thus, development of new PDE inhibitor drugs started at the time when the classical drug discovery process, which was an intimate interplay between pairs of laboratories from medicinal chemistry and pharmacology, was revolutionised by biochemists who introduced new methodologies employing purified enzymes, receptors or cells. The success of drug research programs depended on the understanding of PDEs, which appeared to be peculiarly complex due to the explosion from originally one enzyme in 1958 to over five isoenzymes in 1985, to a protein superfamily that is now known to contain more than 100 members. Since members of the PDE superfamily are expressed in most, if not all, tissues, each desired effect is potentially accompanied by other undesired side effects. In order to analyse and discriminate between desired and adverse effects, a broad understanding of PDE distribution needed to be established. In this historical overview, we concentrate on PDE3, PDE4 and PDE5, where certain inhibitors are approaching or have already reached the goal of approval for use as medications.

2 The Ancestor Theophylline: A Multitalent with Bad Character

2.1 *Asthma and Caffeine*

Therapeutic efficacy of caffeine in asthma patients was originally observed and meticulously described in 1860 by the American physician Henry Hyde Salter in his book “On asthma, its pathology and treatment” (Salter 1860; Persson 1985). Salter suffered from asthma himself and this intimate relationship to the disease resulted in precise observations and quite modern conclusions. He observed that bronchospasms may be induced either by exercise and cold air or by the emanations of domestic cats or hay. He thus characterised hyperresponsiveness as “excessive irritability” of the airways in a lung of “perfect organic health”. He discriminated these acutely occurring phases of dyspnea from more chronic airway disease, which occur as a consequence of bronchitis or of cardiac failure (“cardiac asthma”). He further realised that bronchospasms could be diminished by “sudden alarm, fright, surprise or pleasant excitement” (an effect that is replicated by β -mimetics), smoking dried leaves of *Datura stramonium* (which contains anti-cholinergics that mimic atropine) or by “two morning cups of strong coffee” (which contains a mix of xanthines corresponding to a dose of ~300 mg theophylline (May 1974). In view of today’s knowledge, it seems that Salter’s observations might have been sufficient for the development of various asthma and/or chronic obstructive pulmonary disorder (COPD) therapeutics as they exist today. However, systematic chemistry and pharmacological protocols to develop such drugs for medicinal uses had yet to be developed.

2.2 Xanthines for Medical Remedies

Three xanthines (caffeine, theobromine and theophylline) had been isolated from beans of coffee and cacao and from tea leaves in 1820, 1842 and 1888, respectively, but it was not until 1895 that the chemical structures were analysed and finally proven via chemical synthesis (Fischer and Ach 1895). The first pharmacological investigation using these compounds was performed in an animal model for urine production and was published in 1887 by the pharmacologist Schroeder (1887). He canulised the urether of anaesthetised rabbits and observed that after intravenous administration of theobromine, urine production increased nearly tenfold. This discovery was the birth of the xanthines as “diuretics”. This observation was highly relevant, and its clinical application met an important medical need because it gave relief to patients with leg and lung edema (called “dropsy”) caused by heart failure. This early pharmacological research success stimulated entrepreneurs in chemical industry to launch products containing xanthine mixtures such as “Diuretin” (Knoll is the company that sold that product from 1889), “Agurin” (Bayer 1901) or “Theocin” (Byk Gulden 1902) (Rau 2001). One of the major drawbacks of theobromine and theophylline was their low solubility in water. A freshly employed chemist at Byk Gulden (Grueter 1910) solved the problem by creating a complex containing theophylline and ethylenediamine. This formulation resulted in the first preparation of theophylline for oral and parenteral application and was called “Euphylline”. Heinrich Byk, an entrepreneur who had founded a chemical company in 1873, instantly decided to build a new pharmaceutical factory where Euphylline was produced in various forms, e.g., as an infusion, tablet and suppositories. Although this was a provocation for established pharmacists, who had the privilege and responsibility to mix medications with their own competence, this new drug conquered the market in Europe and USA and was prescribed for the treatment of angina, coronary sclerosis and so-called “hydrops”. By around 1900, 16 of 10,000 Americans in USA received a prescription of one of the available popular xanthine medications. A historical summary is given in Table 2.

Table 2 Early historical milestones of PDE inhibitor research

1820	Isolation of caffeine, theobromine (1842) and theophylline (1888)
1860	H.H. Salter discovers coffee as effective treatment against asthma
1887	W.v. Schroeder determines diuretic function of theobromine in pharmacological experiments
1889	Mixtures of theophylline and theobromine are used as treatment for cardiac insufficiency
1895	O. Langendorff establishes isolated perfused heart
1899	K. Hedbom describes increased contractility of rabbit hearts after caffeine
1912	J. Pilcher finds inotropic function of xanthines
1912	P. Trendelenburg establishes isolated bronchi from the cow
1922	S. Hirsch compares clinical efficacy with pharmacological results and defines xanthines as bronchodilators
1936	G. Herrmann and P. Greene rediscover successful treatment of status asthmaticus with theophylline
1958	T. Rall and E. Sutherland discover PDEs and identify theophylline as PDE inhibitor

Discovery of xanthines, their pharmacological and clinical applications are listed

2.3 *Rationale for Pharmacology*

Driven by the widespread medical use of xanthines and their success in treatment of cardiac insufficiency, the influence of these compounds on cardiac functions were studied in the emerging pharmacological models of the time. Isolated, perfused heart preparations were established by Langendorff (1895), and the xanthine-induced increase of contractile power of the left ventricle was demonstrated using this model (Pilcher 1912; Plant 1914). Furthermore, *in vitro* isolated smooth muscle tissue preparations including blood vessels and segments of intestinal wall (Magnus 1904) were used to demonstrate the effect of xanthines to decrease contractile force and promote relaxation. These effects contributed a further rationale for the use of xanthines for treatment of cardiovascular diseases.

Around 1920, Euphylline was the mainstay for treatment of cardiovascular diseases, and it was Samson Hirsch, MD who shifted the focus to airway pathophysiology (Hirsch 1922). Using suppositories containing a mixture of theobromine and theophylline (ratio 1:2 and called “spasmopurin”), he treated patients experiencing bronchospasms from various origins. He discriminated between young patients with “endogenous asthma” from elderly patients with concomitant “bronchitis”, “circulatory and cardiac insufficiency” and peripheral edema, which were called “cardiac and/or renal asthma”. In both conditions, he observed that Euphylline produced fast relief of dyspnea and bronchospasms as well as a long-term amelioration of the general condition of the patient. He recommends the diuretin/spasmopurin treatment for both (1) “antispasmodic bronchodilatation” and also (2) for “prophylactic treatment” and thereby recognises that a chronic disease underlies the acute symptoms.

Being already familiar with the pharmacological experiments of Trendelenburg using isolated tracheal muscles (Trendelenburg 1912), Hirsch then reinforced his clinical observations by conducting *in vitro* experiments. He measured the influence of the xanthine mixture on isolated bovine bronchial muscle preparations. In the summary of his unique publication on the combined clinical and pharmacological investigations of bronchospasms, he states that (1) theobromine and theophylline are bronchodilators, and (2) bronchi are relaxed independent of their spasmogenic origin. Moreover, he recommends that both drugs should be used not only as “diuretics” but also as “bronchodilators”. This was Hirsch’s only published paper with pharmacological results, and although carefully described, these discoveries did not gain general acceptance or clinical application (May 1974). The reason that such an important discovery was not recognised seems to be twofold: (1) within the title of this paper the terms “asthma” and “theophylline” were not included and thus it may have missed public recognition; (2) apparently, there were no pharmacological societies or clinical opinion leaders available to spread these observations to those in clinical practice. Similar pharmacological results had been obtained by Macht and Ting but without accompanying clinical results (Macht and Ting 1921). It took another 15 years until the bronchospasmolytic potential of theophylline in the status asthmaticus was reinvented in clinical studies

independently by Herrmann and Greene (Herrmann et al. 1937; Greene and Paul 1937). They showed that a very slow intravenous injection of 480 mg theophylline over 2–5 min resulted in a “prompt and persistent relief” from dyspnea. Based on the publication of these investigations, theophylline was promulgated as a bronchodilatory substance and became the mainstay of asthma therapy from 1940 onwards. In the years following 1980, theophylline treatment for asthma was increasingly displaced by inhalative glucocorticoids, but the anti-inflammatory activity of theophylline was still intensively investigated in the 1990s, and today further mechanisms for the action of low-dose theophylline, i.e., as a PI3K inhibitor and HDAC2 activator, are discussed (Barnes 2003a). Since, in addition to these effects, theophylline is also a potent antagonist of the anti-inflammatory A₂-receptor-linked function of endogenous adenosine, it seems that its characteristic therapeutic unreliability is based on the counteraction of its endogenous anti- and pro-inflammatory mechanisms.

2.4 *Narrow Therapeutic Window*

Thus, in the first half of the twentieth century, theophylline had acquired a prominent position in the drug spectrum for use in counteracting various life-threatening disease states. It combined (1) inotropic, (2) diuretic and (3) bronchospasmolytic efficacies. However, this broad clinical spectrum was accompanied by severe, in some instances toxic side effects. Adverse events (AEs) on the cardiac, central nervous system and gastrointestinal functions such as (1) tachycardia, palpitations, tremor and arrhythmias, (2) headache and (3) nausea and vomiting were frequently experienced, but these problems were tolerated since the expectation in the tolerability vs. efficacy ratio in those days was low. Correct dosing was not possible because of the lack of knowledge of pharmacokinetics for these drugs, and the dose was determined by trial and error. We know today that proportionality of dose to blood concentration of theophylline is unusually weak (Ohta et al. 2004) and that even at low doses of 2×200 mg/day blood concentrations of >15 mg/l (in 13% of patients) followed by toxic AEs may occur. The most severe and frightening AEs of theophylline were seizures, which had already been published by Allard (1904). He describes two patients who were repeatedly injected with a relatively small dose of 300 mg theobromine. They had been rescued from dyspnea, increased their cardiac contractility, and their general condition had been significantly ameliorated. In spite of this considerable beneficial effect of the medication, these two patients suddenly and surprisingly were attacked by “epileptic convulsions” and died a few minutes later. This alarming finding was instantly confirmed in toxicological experiments with dogs and rabbits. Similar cramp phenomena were observed after diuretin administration when dogs died and a “lethal dose” of 500 mg/kg was evaluated. This dose was far greater than the critical doses in man (500 mg per patient), and a clear correlation from dog to man could not be drawn. Today, it is well known that blood concentrations established after a constant dose of

theophylline show massive deviations among individuals. The Ohta study which included nearly 3,800 patients underlined this unusually shaky ratio, and no significant relation between dose and AEs could be found (Ohta et al. 2004). Consequently, in clinical studies patients had to be preselected for AEs, and for the practitioner, theophylline had to be individually dosed after measuring blood concentrations (Wilkins et al. 1984) and even then the probability of AEs was high.

2.5 Theophylline as Tool for PDE Research

The identification of theophylline as a PDE inhibitor emerged in the initial experiments of Sutherland and Rall in 1957 when they opened the world of cAMP signalling (Rall and Sutherland 1958). They investigated hormone action on glycogenolysis in broken cell preparations and found that caffeine inhibited the basal, non-activated form of glycogen phosphorylase. They reasoned that addition of caffeine to the adrenaline-stimulated interconversion test might improve the detection of the activated phosphorylase. In contrast to their expectation, they observed a synergy of caffeine with adrenaline or glucagon in activating phosphorylase. From this result, they consequently hypothesised that caffeine might inhibit either the activity of the agent that destroyed their “heat-stable factor” or the phosphorylase-phosphatase (Butcher 1984). Later, when this heat-stable factor was identified as cAMP – the “golden bullet” for second messenger signalling – the enzymatic activity for breakdown was determined to be “phosphodiesterase activity” and concomitantly, caffeine and theophylline became the first recognised PDE inhibitors (Rall and Sutherland 1958). In the following years, the potentiation of adrenaline effects was shown in pharmacological and biochemical assay systems: inotropic responses in isolated perfused hearts (Rall and West 1963) and likewise in other tissues.

3 Pharmacological Models Need to Be Analysed Biochemically

3.1 Different Inhibitory Profile Indicate Multiple Enzymes

Papaverine was isolated in 1848 and introduced as the second PDE inhibitor after the xanthines. Pharmacological activity of both types of compounds were compared in a variety of contractile preparations including heart, vascular and intestinal smooth muscle, isolated bronchi and also in metabolic functions such as lipolysis of isolated fat cells or glycogenolysis in liver tissue. Papaverine functioned as an efficacious smooth muscle relaxant and was denominated a “direct vasodilator”, but it showed comparably less inotropic activity in the heart or enhancement of lipolysis or glycogenolysis. Theophylline was weaker in potency and in vascular and intestinal muscular preparations where it predominantly potentiated adrenaline

effects, but it was equally effective in each system studied. These early observations of the different pharmacological and biochemical profiles of the PDE inhibitors was observed by several authors (e.g. Poech and Kukovetz 1971) and gave rise to the view that there must be multiple PDE enzymes and that different tissues may contain different PDEs.

3.2 Column Chromatography Profiles of PDEs and Development of the First Generation of PDE Inhibitors

Up to 1985, a variety of pharmacological models for PDE inhibitor research had been established, and the prominent ones are listed in Table 3. In order to understand the interference of the available old and new substances, a biochemical analysis clarifying PDE content and diversity of all these models appeared to be inevitable. Thompson and Appleman provided key pioneering experiments by successfully using anion-exchange chromatography for separation of a number of

Table 3 Isolated organs and tissues available in 1985 for investigation of functions of PDEs 3, 4, 5

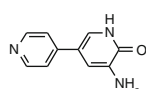
Preparation	Pretreatment	Response	PDE
Aorta, rat	L-Phe	Relaxation	
Intact			PDE5 > PDE4
Denuded			PDE3 > PDE5
Pulmonary artery, rat	L-Phe	Relaxation	
Intact			PDE5
Denuded			PDE5
Coronary artery, gp	L-Phe	Relaxation	
Intact			PDE5
Denuded			PDE5
Perfused heart, gp	Spontaneous	Contractility dp/dt	PDE3
		Coronary flow	PDE5 > PDE3
		Heart rate	PDE3
		LV pressure	PDE3
Left atrium, gp	Electrical stimulation	Force of contraction	PDE3
Tracheal rings, gp			
Untreated	Spontaneous		PDE3, 4, 3/4
Sensitised	OVA challenge		PDE4
Lung strips, gp	Histamine, carbachol	Relaxation	PDE3

Isolated organ and tissue preparations have been developed from the end of the nineteenth century. Functional analysis was based on isometric force transduction for either contraction or relaxation. The preferred species for each preparation is mentioned (gp = guinea pig) and the pre-treatment to reach a contracted state ready for relaxation by PDE inhibitors is given
OVA ovalbumin, *L-Phe* L-phenylephrine

PDE isoenzymes (Thompson and Appleman 1971). Thompson and his associates separated PDEs from cardiac and cerebellum tissues (Thompson et al. 1979), and Hidaka and Polson resolved those of platelets and canine trachea (Hidaka and Asano 1976; Polson et al. 1982). Platelets and cardiac tissue each revealed three peaks of PDE activity, whereas in canine trachea five different peaks of PDE activity were resolved. The PDEs in each of the peaks were characterised with enzymologic criteria such as (1) substrate specificity (cAMP/cGMP), (2) substrate affinity and (3) calmodulin and cGMP activation, but the data were hardly comparable and, even worse, every author used his own nomenclature. The discrimination of five different PDE classes emerged only after the separation methods were more refined and more selective tools (activators/inhibitors) were applied for characterisation of the peaks. Much of the confusion concerning the PDEs was largely ended, and each PDE peak of tissue-specific elution pattern could be attributed to this system. However, other complications such as proteolysis and expression of myriads of alternative splice variants in some families continued to complicate understanding of these enzymes. The publication of Reeves in 1987 (Reeves et al. 1987b) clarified that the cardiac peak III can be further separated into two cAMP-hydrolyzing PDEs where the earlier eluting peak is the highly cAMP specific rolipram-sensitive (now known as PDE4) and the later eluting cGMP-inhibited cAMP-PDE (now known as PDE3). This publication marks the time point when the system of PDE1–PDE5 with their typical enzymological characteristics became established in most laboratories that were engaged in PDE research (Weishaar et al. 1985; Nicholson et al. 1989; Schudt et al. 1991a, b, c; Torphy and Cieslinsky 1989). The elegant concept of a protein superfamily of six PDE families with each family containing several members was composed by Joe Beavo (1988), and many later publications along with the contributions of other pioneers in the field (Marco Conti and Rick Heaslip) unfolded the whole world of >60 PDEs in 11 families (Beavo et al. 1994; Conti and Beavo 2007). The enormous biodiversity of these key regulatory enzymes and the view of their distribution in different tissues underlined the rationale for searching for new drugs with defined selectivity for one or more PDE families or subtypes.

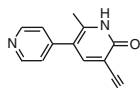
Around 1985, a pool of around 30 PDE inhibitors with weak potencies and selectivities was available; these have been listed and their chemistry has been described extensively in excellent reviews of that time (Weishaar et al. 1985; Torphy and Udem 1991; Nicholson et al. 1991; Beavo 1988). Prominent representatives and important tools for research progress at that time were SKF 94120, SKF 94 836, milrinone and motapizone for PDE3, rolipram and Ro 20-1724 for PDE4 and zaprinast for PDE5. Milrinone, rolipram and zaprinast had already been studied in patients as cardiotonics, antidepressants and bronchodilators, respectively (see Table 1). Due to insufficient safety or low therapeutic efficacy, these developments had to be discontinued. The positive aspects of these early and engaged trials was the demonstration that therapeutic efficacy in principle is possible and can be improved. Further, the recognition of AEs and of the necessity to study and understand their biochemical mechanisms was of considerable value for future research (for details of rolipram studies, see following chapters). On the

PROTOTYPE

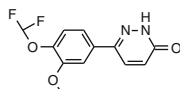


Amrinone

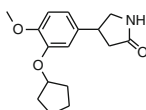
ADVANCED PDE INHIBITORS



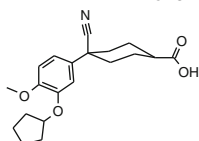
Milrinone



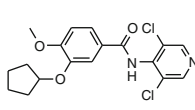
Zardaverine



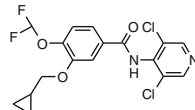
Rolipram



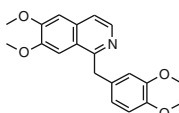
Cilomilast



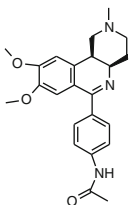
Piclamilast



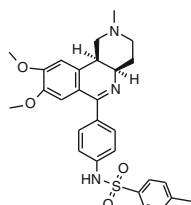
Roflumilast



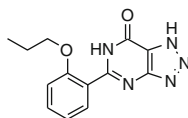
Papaverine



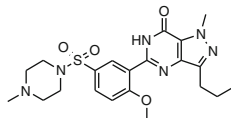
Benafentrine



Tolafentrine



Zaprinast



Sildenafil

Fig. 1 Chemical structures of prototypic and advanced PDE inhibitors

contrary, these compounds served as lead structures for chemical optimisation (for some examples see Fig. 1) and were useful tools for PDE research especially for evaluating the relevant PDE functions in intact cells and tissues.

3.3 Six Lessons About PDE Inhibitor Function in Isolated Organs

3.3.1 Potentiation of Adenylate and Guanylate Cyclase Agonists

Isolated organs such as aorta, pulmonary artery, intestinal segments from ileum and perfused heart had been established and used for characterisation of mediators, contractile agonists and antagonists since the beginning of the last century. In vascular preparations, papaverine differed from the weaker theophylline because it was a very potent relaxing agent by itself and was therefore called a “direct vasorelaxant” (Poech and Kukovetz 1971). Theophylline, however, seemed to be more efficacious in perfused heart preparations, isolated papillary muscle or isolated atria (Rall and West 1963). Both compounds relaxed the various preparations (1) without

direct interaction with agonists at the receptor, (2) apparently acting intracellularly and (3) potentiating relaxation by adrenaline, histamine, adenosine, vasoactive intestinal peptide (VIP) or dopamine. This agonist-potentiating effect became a characteristic mechanism of PDE inhibitors, which amplify physiological signals and thus might be therapeutically relevant.

The new PDE3 inhibitors such as amrinone and milrinone showed the same inotropic spectrum of changes in contractility especially on the force development and with minor effects on rate of contraction (Honerjäger et al. 1981). With time, the isolated perfused heart preparation was established as the preparation to detect PDE3 inhibitor efficacy, and additionally, later demonstration of PKA activation gave further evidence for the interference with the cAMP-mediated dilatory pathway (England and Shahid 1987). In contrast, coronary flow in constant-pressure perfused heart preparations was enhanced by the PDE5 inhibitor zaprinast (M&B 22,948), and its potentiation by addition of nitric oxide (NO)-donating agents was described in 1979 by Kukovetz and later by Lorenz and Wells (Kukovetz et al. 1979; Lorenz and Wells 1982). Katsuki had demonstrated in 1977 that nitroglycerin stimulated guanylate cyclase (GC) followed by cellular increase of cGMP whereby elevated cGMP relaxed coronary and pulmonary artery smooth muscle tension (Katsuki et al. 1977). Therefore, it was reasonable that inhibition of cGMP-degrading PDE5 evoked relaxation in these vascular preparations. In 1993, human pulmonary artery smooth muscle was shown to contain PDE3 and PDE5 as predominant PDE activities, and the efficacies of PDE3 and PDE5 inhibitors were additive (Rabe et al. 1994). This synergy with agonists of cyclases and additivity of different PDE inhibitors was shown in a variety of smooth muscle preparations and cells. The synergism of NO with zaprinast in lowering pulmonary artery pressure (PAP) in lambs with pulmonary hypertension (PHT) was shown (Thusu et al. 1995), and these *in vitro* and *in vivo* observations provided the first allusion for applying PDE5 inhibitors in treatment of PHT.

3.3.2 Even Isolated Organs Can Be Denuded

Another lesson taught by isolated organs was the discovery of the effects of “denudation” of vascular preparations. Acetylcholine (ACh)-induced relaxation of pulmonary artery was abolished after removal of the endothelium. Furthermore, the archetypical GC inhibitor, methylene blue inhibited relaxation of the denuded muscle to NO-releasing agents. These results demonstrated that upon ACh stimulation the endothelium releases NO [also known at the time as endothelium-derived relaxing factor (EDRF)], which in turn activated GC. GC was inhibited by methylene blue and no longer stimulated the cGMP pathway (Gruetter et al. 1980). Thus, different mechanisms underlying relaxation mediated by changes in the endothelium and those mediated by events in the target organ need to be discriminated since complete and denuded preparations differ in sensitivity towards different PDE inhibitors (Table 3).

The most innovative example of the NO/cGMP pathway with therapeutic and lifestyle implications was discovered by Ignarro when he determined that NO was the mediator for penile erection. NO is released by electric stimulation from nitrergic nerves in corpus cavernosum and thereby relaxes arterial and trabecular tone in this muscular organ (Ignarro et al. 1990). NO again stimulates cGMP production, and this local concentration may be amplified in the presence of PDE5 inhibitors. The potentiation of NO signalling by PDE5 inhibitors in the corpus cavernosum was described and became the standard preparation for PDE5 inhibitors for fast-onset and long duration penile erection (Knispel et al. 1992).

3.3.3 Additive Contribution by Several Different PDE Inhibitors

In asthma, a reversible contraction of large and medium airways is evoked by irritants, and mediators such as histamine, ACh, thromboxane, leukotrienes or allergens. Other mediators such as VIP, NO and PGE2 and pharmacological agents such as β 2-mimetic drugs counteract this constriction and relieve the dyspnea attack. This function is mimicked in vitro in isolated tracheal rings from the guinea pig which were the technical improvements of the tracheal muscles from larger animals such as the cow or dog. These rings develop a spontaneous contraction which can be further enhanced by agonists such as histamine, leukotrienes or carbachol (Torphy et al. 1988). The unselective PDE inhibitor theophylline at 100 μ mol/l evokes a complete relaxation of these rings comparable to that achieved by treatment with isoproterenol. Partially selective PDE inhibitors, such as PDE3 inhibitor CI930, start relaxing at much lower concentrations but a flat and sometimes biphasic concentration–response curve is obtained which does not allow determination of exact ED50 values. It was Harris et al. (1989) who finally solved this problem elegantly by comparing the substance activities in the mechanical and biochemical assessment. He chose CI930 and rolipram which inhibit PDE3 > PDE4 and PDE4 > PDE3, respectively, both exhibit an approximately 100-fold selectivity ratio for inhibition of these PDEs in isolated PDE fractions from guinea pig trachea. He then determined the threshold concentrations for complete inhibition of PDE3 by CI930 and PDE4 by rolipram. In the pharmacological experiment, he then pretreated the tracheal preparation with these threshold concentrations, thereby blocking the action of one of these PDEs, and then measured a concentration–response curve for the other inhibitor. From the resulting monophasic curves, correct ED50 values for these (not completely) selective compounds could be evaluated. Thus, he answered two questions (1) the contribution of two different PDEs in one tissue to relaxation and (2) the additive fractional efficacy of PDE3 and PDE4 inhibition of dual-selective inhibitors, which may offer therapeutical benefit by bronchorelaxation.

3.3.4 Predictions Are Not Always Unequivocal

In the therapeutically relevant tissue of human bronchi, the contributions of PDE3 and PDE4 inhibitors were assessed in biochemical and pharmacological experiments by various investigators, however, with results that varied substantially. Rabe et al. (1993) found no significant contribution of the PDE4 inhibitor to relaxation in bronchi of human origin. Similarly, low efficacy and potency of rolipram was shown by Torphy et al. (1993). In contrast, in the papers of Cortijo et al. (1993), Tomkinson et al. (1993), Naline et al. (1996), PDE4 inhibitors rolipram and pliclamilast showed high potency and efficacy in relaxation of human bronchi. These contradictory results demonstrate that *in vitro* experiments do not always provide unequivocal answers. Pretreatment of the preparations and the presence of endogenous agonists (in this case leukotrienes, adenosine) may have considerable influences. The above conflicting results were reconciled by experiments conducted in the clinic. In clinical studies, neither of the two PDE4 inhibitors (cilomilast in doses of 15 mg in COPD patients) (Grootendorst et al. 2003) nor roflumilast at doses of 500 and 1,000 µg in asthma patients (Engelstaetter et al. 2005) evoked any acute improvement of FEV1 (a measure of bronchodilation).

3.3.5 Disease-Relevant Models Are Possible on the Level of Isolated Organs

The influence of an allergic challenge on tracheal contraction can be determined if the tracheal tissue is derived from animals that have been previously sensitised. Thus, isolated tracheal rings from guinea pigs that had been sensitised with ovalbumin (OVA) were established in parallel to routine *in vivo* experiments with non-sensitised animals. Contraction in this preparation is induced by addition of the allergen (OVA) in order to mimic the bronchoconstriction that occurs during an allergic episode (preparations that have been denuded loose antigen sensitivity). Preincubation with a PDE4 inhibitor was shown to maximally inhibit the resulting antigen-induced contraction by 80%. PDE3 inhibition was not effective against mediator release from the sensitised tracheal mucosa (Underwood et al. 1993; Bundschuh et al. 2001). Similarly, very early non-specific inhibitors had been found to suppress histamine and leukotriene release from lung strips of allergic animals or men (Orange et al. 1971), and the PDE4 specificity was established by rolipram inhibition of histamine release from human basophils (Frossard et al. 1981).

3.3.6 PDE Inhibitors Do Not Always Inhibit via PDE Inhibition

In order to demonstrate the functional and causal relationship between biochemical and pharmacological IC₅₀ and ED₅₀, these data can be examined in correlation diagrams for several available PDE inhibitors. For PDE3 inhibitors, a sufficient correlation was obtained for the potency of PDE3 inhibition and the corresponding

relaxation elicited (Harris et al. 1989). This result demonstrated (1) that PDE3 inhibition contributes to relaxation and (2) that inhibition of PDE3 is similar in the cell-free system and in the intact cell. On the contrary, although PDE4 inhibition contributed significantly to relaxation, no obvious correlation was obtained. This indicates that the IC₅₀ for inhibition of isolated PDE4 does not quantitatively reflect the effect of PDE4 inhibition in the intact tissue. This result was surprising and the lack of correspondence of the effect of an inhibitor on isolated PDE4 with the effects of the inhibitor on PDE4 in a tissue, respectively, was a challenging phenomenon for optimising the effects of new compounds targeting PDE4 function. Interestingly, a much better correlation was obtained if, instead of enzymatic IC₅₀, the IC₅₀ for competition with binding of radioactive rolipram to the PDE4 was used. This raised the hypothesis that the binding competition is the better measure for the affinity of the inhibitor to PDE4 enzyme in the tracheal muscle (discussion in Sect. 4.5).

4 Isolated Cellular Systems

4.1 *Human Platelets Offer Easy Determination of PDE3 and PDE5 Inhibitors*

Human platelets offered at least four important advantages as an analytical tool. These cells and their enzyme content were of (1) human origin, (2) easy to prepare and (3) represented a homogeneous cell preparation. Moreover, aggregation induced by ADP or collagen was an established routine measurement, and theophylline and papaverine completely inhibited aggregation indicating a relevant function of PDEs. This important regulatory function was further improved by inhibitors of aggregation such as forskolin, adenosine or β_2 -agonists representing AC activators. Hidaka had already shown in 1976 that two prominent peaks of PDE activity, which were cGMP specific (peak I) and one cAMP specific (peak III), respectively, could be separated from platelets (Hidaka and Asano 1976). Later peak III was identified as cGMP-inhibited and cAMP-hydrolysing PDE3, which was undistinguishable from soluble PDE3 in cardiac tissue (MacPhee et al. 1986). Thus, the crude extracts of platelets offered two easily distinguishable PDEs, and in addition, a common function related to both PDEs could be measured. The correspondence of the effect of a PDE inhibitor on the isolated enzymes and its effect in the intact platelet could be confirmed in a perfect correlation of both data sets (Schudt et al. 1991c). The relationship between an increase in platelet cGMP, which is followed by an increase in cAMP due to cGMP-inhibition of cAMP breakdown at the catalytic site of PDE3 was first demonstrated in 1990 (Maurice and Haslam 1990). The presence of a third PDE fraction was indicated in the elution profiles and identified as PDE2 (Schudt et al. 1991a, b, c), and the interaction of a cGMP-stimulated and a cGMP-inhibited PDE could be studied functionally (Dunkern and Hatzelmann 2005).

4.2 *The PDE4-Regulated Inflammatory Cells: Neutrophils, Eosinophils, Basophils, Monocytes*

Human neutrophils represent proinflammatory cells, which can be isolated easily and routinely from human blood. After comparing activators such as opsonised zymosan or C5a it appeared that fMLP was the most reliable activator for degranulation, secretion and release of reactive oxygen species (ROS). Chromatography of neutrophil extracts (which at that time had not been carefully separated from adherent platelets) showed one prominent peak, which appeared to be highly sensitive to rolipram and Ro 20-1724 (Schudt et al. 1991a). Quantitation of ROS release from neutrophils with the cytochrome C method was introduced by Lad et al. (1985), who demonstrated the reduction of the oxidative burst by prostaglandins, isoproterenol and IBMX. Analysis of the relationship and cellular inhibition by this method revealed correlations between biochemical characteristics of PDE4 inhibitors (IC₅₀ of PDE4) and the pharmacological effects of these inhibitors on ROS release in neutrophils (EC₅₀ of ROS) (Schudt et al. 1991c) and eosinophils (Barnette et al. 1995b). These published correlations, however, were no longer true when from the beginning of the 1990s, the detection method for ROS release was changed to the much more sensitive chemoluminescence measurements (Nielson et al. 1990; Schudt et al. 1991b).

Eosinophils were known to be crucially important in asthma, since asthma was denominated as “eosinophilic bronchitis” (Kay 1987). Eosinophils were usually isolated from guinea pigs, which frequently exhibited an endogeneous eosinophilia. Thus, these proinflammatory cells became available for testing new inhibitors and were investigated in the group of PJ Barnes (Dent et al. 1990, 1991). It emerged that (1) the oxidative burst of eosinophils was sensitive to PDE4 inhibitors, and since PDE4 was the only cyclic nucleotide-degrading enzyme in these cells, other secretory parameters such as (2) LTC₄ and (3) eosinophilic cationic proteins (ECPs) were also determined to be affected by PDE4 inhibition (Hatzelmann et al. 1995; Tenor et al. 1996).

Basophils and mast cells are responsible for histamine and LTC₄ release (slow-reacting-substance of anaphylaxis, SRS-A at that time). Strips of human lung tissue can be stimulated to contract by IgE, and it was already observed in 1972 in the groups of Liechtenstein and Austen that cAMP analogs, PGE₂ or theophylline could inhibit this release which resembles the “relieving” mechanisms counteracting the primary allergic attack (Liechtenstein and Margolis 1968; Kaliner et al. 1972). Later, in basophils from atopic patients PDE inhibitors reduced mediator release, enhanced cAMP concentration and the cAMP/PKA involvement in the secretory mechanisms was demonstrated (Marone et al. 1987). The reduction of antigen-induced histamine release by PDE4 inhibition was described by Kleine-Tebbe in atopic basophils (Kleine-Tebbe et al. 1992). Mast cells would have been the desired model for allergic inflammation, but due to their complicated isolation the model of lung strips from atopic animals was used instead in many laboratories (see Sect. 3.3.6).

Table 4 Isolated cell models for studies of asthma-related processes in 1990

Model	Preferred stimulus	Response	PDE
Platelets	Collagen, ADP	Aggregation	3,2,5
Neutrophils	fMLP	ROS, LTB ₄ , HNE, CD11b	4
Eosinophils	C5a	ROS, IL-5, LTC ₄ , ECP	4
Monocytes	LPS	TNF α , IL-10, LTB ₄	4
Basophils	Allergen	LTC ₄ , His, PGD ₂ , GMCSF	3,4,3/4
T-cells	Anti-CD3/CD28	IL-4, IL-5, IFN γ , proliferation	3,4,3/4
Macrophages	LPS	TNF α	3,4,3/4
Dendritic cells	LPS	TNF α	3,4,3/4

Cell preparations from human origin were the preferred model system for characterising future drug candidates. Cell populations were enriched from human blood by multi-step separation procedures, which were optimised over the years and ended up in one-step magnetic cell sorting or culture-derived cells

ROS reactive oxygen species, HNE human neutrophil elastase

Monocytes (MCs) are the circulating precursor cells of macrophages. Before the routine techniques for obtaining highly purified leukocyte populations by elutriation and magnetic cell sorting were established (Gantner et al. 1997a), less sophisticated but highly time-consuming methods were used. MCs were obtained from human blood by gradient centrifugation and the resulting mixture of 80% lymphocytes and 20% MC could be investigated (Thompson et al. 1976; Semmler et al. 1993). The alternative separation procedure was adherence to plastic dishes and separation from non-adherent cells (Schade and Schudt 1993). Upon stimulation with lipopolysaccharides (LPS), MCs release a variety of proinflammatory factors including hydrolytic enzymes, growth factors, lipid mediators, ROS and cytokines. TNF α and the matrix-degrading enzymes such as matrix metalloproteases (MMPs) for collagen and elastin are prominent mediators of inflammation in asthma, rheumatoid arthritis (RA) and COPD. Inhibition of TNF α generation in human and mouse MCs was demonstrated to be effectively reduced by PDE4 inhibitors in 1993 (Semmler et al. 1993; Schade and Schudt 1993). MC PDE composition was originally analysed by Thompson and a considerably homogeneous peak of PDE4 was revealed by chromatography (Thompson et al. 1976). A summary of isolated cell models as they were available at about 1990 to explore asthma-related processes is given in Table 4.

4.3 T-Cells, Macrophages and Dendritic cells for Dual PDE Inhibitors

T cells release several cytokines which contribute to the inflammatory process in various diseases such as asthma, RA, intestinal bowel disease (IBD), multiple sclerosis (MS) or COPD. T-cell responses upon immunological stimulation such as cell activation, cytokine secretion and clonal proliferation depend on the cAMP/

PKA pathway, and this was observed in a variety of early studies (Kammer 1988). An analysis of isolated T cells comparing PDE inhibitor sensitivity of secretory functions and blastogenesis with PDE isoenzyme profiles was first performed by Robicsek showing that both PDE3 and PDE4 are present in T cells (Robicsek et al. 1989, 1991). T-cell isolation in those days was a complicated, time-consuming multi-step procedure with low yield and the risk for activating quiescent cells. Later, a standardised technique for cell isolation and measurement of intracellular PDE activities was developed (Gantner et al. 1997b, Schudt and Tenor 1996), which by using selective inhibitors, allowed an approach for determining PDE isoenzyme profiles in isolated CD4+ and CD8+ T cells or macrophages (Tenor et al. 1995b, c). Functionally, it was again demonstrated that cytokine release was partly inhibited by either PDE3 or PDE4 inhibitors, whereas complete inhibition was observed in the combined presence of both inhibitors or dual-selective PDE3/4 inhibitors such as zardaverine. This indicated that PDE3 and PDE4 participate in regulation of the cAMP pathway in T cells.

Alveolar macrophages showed similar inhibitory patterns (Schade and Schudt 1993; Tenor et al. 1995c), but since they were difficult to obtain they were differentiated in cell culture together with dendritic cells from blood MC (Gantner et al. 1997a). In parallel to phenotypic differentiation, PDE1 and PDE3 activities were augmented whereas PDE4 activity – the major activity in MC – declined, and MC-derived macrophages acquired the identical PDE activity profile as found for alveolar macrophages. Functionally, simultaneous inhibition of PDE3 and PDE4 was most effective in inhibition of TNF α release from both MC-derived cell populations (Gantner et al. 1999).

4.4 Does Exposure of Inflammatory Cells to β -Mimetics Evoke Hyperreactivity?

Absolute PDE activities in cells and tissues were of considerable interest; however, all these determinations of cellular enzyme activities were based on defined substrate concentrations and enzymological standard conditions. PDE profiles as described in many papers were useful for comparing different tissues and for explaining the functional effects of mono- or dual-selective PDE inhibitors. On the contrary, it was obvious that PDE activities in situ were regulated at various levels: (1) substrate concentrations and kinetic behaviour, (2) concentration of competitors and endogenous inhibitors, (3) regulation by phosphorylation and/or other modifications, (4) intracellular compartmentalisation and membrane association and (5) degradation and de novo synthesis. An upregulation of PDE4 could be a sufficient explanation for an enhanced activity state of PDE4-containing cells. Indeed, such enhanced PDE4 activity levels in MC and/or lymphocytes from atopic patients were described by Hanifin in several papers (Holden et al. 1986). Unfortunately, these claims were not reproducible by others (Gantner et al. 1997a, b).

An upregulation of PDE4 was first described by Torphy in a monocytic cell line U937 (Torphy et al. 1992a). Incubation of these cells with salbutamol and rolipram for 3 h was shown (1) to increase intracellular cAMP, (2) to activate PKA and (3) to enhance PDE4 activity two- to threefold. After withdrawal of the stimulating conditions, PDE4 activity returned to the basal levels within a 3 h period. Thus, a feedback mechanism reacting to a sustained increase of cAMP was discovered. Similar experiments were performed with the human keratinocyte cell line HACAT, where a 6 h exposure to salbutamol raised PDE4 activity about threefold (Tenor et al. 1995a). The cAMP increase in these “induced cells” in response to incubation with β -receptor agonists was now considerably reduced, which might be of relevance for the situation in airway diseases where there is continuous exposure to long-acting β -mimetics.

In primary human MCs, a two- to threefold induction of PDE4 activity was demonstrated by Verghese and the upregulation of the splice variants PDE4A5, 4B2 and 4D3 was shown on the level of protein analysis (Verghese et al. 1995). Analogously, in peripheral T cells, the fenoterol-induced upregulation of PDE4A4, 4D1, 4D2 and 4D3 was shown by expression data and western blot analysis (Seybold et al. 1998). In view of these results, a continuous exposure of inflammatory cells in a patient’s lung to long-acting β -mimetics should increase the reactivity to irritants, allergens, stimulators and inflammatory mediators. In conclusion, the finding that (i) β mimetics drive inflammatory cells to an enhanced hyperresponsive state and that (ii) PDE4 inhibitors reduce this up-regulated activity may provide a further rationale for the therapeutic use of PDE4 inhibitors.

4.5 Correlation Diagrams: How Do Biochemical Data Correspond to Pharmacological Results?

T cells and smooth muscles contain a complex mixture of PDE isoenzymes and the actual activity of these different isoenzymes can only be roughly approached by biochemical methods. In order to develop and optimise selective and efficacious PDE inhibitors it was essential to know the participation of a certain isoenzyme in the regulation of defined cell responses. One possibility used to analyse the involvement of a PDE isoenzyme in cellular activity is the correlation of the two parameters that reflect action of PDE inhibitors, i.e. (1) EC₅₀ of the cellular response to treatment with a given inhibitor and (2) IC₅₀ of inhibition of the isolated enzyme by that compound. If the correlation coefficient for a variety of different substances equals 1, then it can be assumed that this very PDE isoenzyme participates in regulation of this cellular function. For platelet aggregation and tracheal relaxation, a correlation with PDE3 inhibition by various weakly selective substances was already mentioned in Sects. 3.3.6 and 4.1 showing that PDE3 was causally and quantitatively involved in the inhibition of functional responses (Schudt et al. 1991c; Harris et al. 1989). Therefore, the inhibitor potency measured

in vitro corresponds to the inhibitor potency at the same enzyme in the cellular environment.

The prototypic PDE4 inhibitor rolipram was a relatively weak inhibitor of isolated PDE4 with an IC₅₀ of ~100 nM (Schudt et al. 1991b). However, in rats in vivo it was found to be pharmacologically active at blood concentrations below 100 nM and correspondingly, a high affinity binding site in rat brain membranes with an IC₅₀ of ~3 nM was identified (Wachtel and Schneider 1986; Schneider et al. 1986). This binding site was identified as a membrane-bound PDE4 and thus it was concluded that the catalytic IC₅₀ and the high affinity binding coexist in the same protein. This hypothesis was verified when the coexistence of both affinity sites in one enzyme preparation was reported (Torphy et al. 1992b), indicating that this enzyme may adopt two different conformations. Importantly, it was reasoned that the centrally mediated effects such as antidepressant activity but also the clinical AEs such as nausea and vomiting should be mediated by the “high affinity state” whereas the “low affinity state” or catalytic site was observed in the enzymological measurement with an as yet undetermined pharmacological correlate. With regard to pharmacological and PDE4-mediated test systems such as isolated organs or isolated cell systems, it was of crucial importance to determine whether effects in these models were related to the IC₅₀ for catalysis or to IC₅₀ for high-affinity rolipram binding site. When generating new substances, however, it was important to know whether or not they discriminate between the sites. It was shown in 1987 by a group at Pfizer that the ratio between these two parameters (inhibition of radiolabeled rolipram binding or catalysis) may vary for different substances and that the ratio of the rolipram-binding affinity to potency of enzyme inhibition was about 200 for rolipram but 0.2 for papaverine (Russo et al. 1987). Thus, it was evident that compounds with mutual preference for the binding site or the catalytic site already existed and that new substances with selectivity for either high affinity or the low affinity site could be designed.

The pharmacological data and correlations demonstrated an association with the high affinity site for (1) ROS release from neutrophils (Barnette et al. 1996), (2) cAMP accumulation in eosinophils (Souness 1996), (3) relaxation of guinea pig tracheal smooth muscle (Harris et al. 1989), (4) H⁺-secretion in gastric glands (Barnette et al. 1995a) and (5) behavioural responses in rats as well as antidepressant effects in man (Schmiechen et al. 1990). Alternatively, an association with inhibition of the lower-affinity catalytic site function of PDE4 was published for (1) TNF α release from MC (Barnette et al. 1996; Souness et al. 1996) and (2) IL2 secretion from mouse splenocytes (Souness et al. 1997).

Thus, differentiation between the “high affinity” and “low affinity” states on the level of the enzyme was operationally defined since the early 1990s, although mechanistically the differences in the two states were not completely understood. Biochemical and cellular models emerging between 1985 and 1995 could detect the potency of new substances in both enzyme conformations, and it then became possible to characterise new substances and to modify them in either direction (see Sect. 6.4).

5 The Path to the Clinic with Cardiovascular Models

5.1 *New Drugs with PDE Inhibitor Function for Cardiac Rejuvenation*

Around 1960, the treatment of heart failure with cardiac glycosides as well as theophylline had acquired an established place in the clinic. However, both medications suffered from (1) a narrow therapeutic window, (2) unwanted AEs such as tachycardia and (3) risk of digitalis intoxication. Research at Sterling-Winthrop and Warner-Lambert had revealed a variety of new compounds with the leading compound amrinone in 1975 (Farah and Alousi 1978; Alousi et al. 1979). Pharmacological studies were performed predominantly in models of rats and dogs which were used for determination of blood pressure, heart rate, cardiac contractility and urine production (Alousi et al. 1979). In anaesthetised animals, substances were administered intravenously (iv) whereas in conscious models oral administration (po) was possible and cardiovascular parameters were not disturbed by anaesthesia. These cardiovascular models including modifications in cats and guinea pigs had been previously established for the development of different classes of antihypertensives such as α - and β -adrenoceptor antagonists, Ca^{2+} -antagonists and ACE inhibitors. For investigation of PDE3 inhibitors, these models could be used without any major changes (Table 4).

For amrinone, the in vivo dog model showed an increase in contractile force of the left ventricle as had been predicted from the measurements in isolated organs (Alousi et al. 1979). In the conscious dog dose-escalating studies showed that pharmacological effects of amrinone started at 0.1 mg/kg iv, side effects were observed from 3 mg/kg iv and the highest non-toxic dose was 10 mg/kg iv or 50 mg/kg po. These data demonstrate a therapeutic index (TI) of about 100 which represents a considerable improvement over cardiac glycosides with TI of 2–3. Amrinone could be administered orally and with an iv/po efficacy ratio of 1:2 revealed a satisfying oral bioavailability. Initial pioneering clinical studies showed beneficial efficacy in patients (LeJemtel et al. 1979; Benotti et al. 1978), which were later repeated with a variety of different PDE3 inhibitors (Weisshaar et al. 1983; Wetzel and Huel 1988). However, in the PROMISE study for chronic treatment of severe chronic heart failure with milrinone within a time period of maximally 12 months the arrhythmogenic potential of PDE3 inhibitors was finally ascertained. In this study, an increase of mortality and morbidity of 28% was reported (Packer et al. 1991). Consequently, further clinical development of PDE3 inhibitors as cardiotonics was terminated and the reasons for this deleterious effect will be discussed in two subsequent chapters in this volume (chapter by Y. Liu, et al, and chapter by M. Movsesian and R. Kukreja). A partial revival of PDE3 inhibitors occurred when ibudilast was approved and launched for intermittent claudication (Kumar and Bhattacharya 2007).

5.2 Potentiating NO Action with PDE5 Inhibitors

For the fascinating success of the prototypic PDE5 inhibitor sildenafil, three paths to discovery had to meet: (1) basic and clinical concepts for vasodilation, (2) development of potent and efficacious compounds and (3) the discovery of NO and its role in male sexual function.

The vasodilating properties of papaverine in coronary arteries and the potentiation of nitroglycerine, which had been used for more than 100 years for treatment of coronary heart disease, had been described originally in 1979 by Kukovetz in the isolated organ model (Kukovetz et al. 1979). It was further clarified that the action of nitrates and also the vasodilating hormone atrial natriuretic peptide (ANF) was mediated by an intracellular increase in cGMP. The cGMP-hydrolysing enzyme had been isolated and described by Francis and Corbin (Francis et al. 1980), and these pieces of knowledge led to the hypothesis that PDE inhibitors of this enzyme being more selective and potent than papaverine should have potential as drugs for treatment of coronary artery disease and vascular maladies. With zaprinast, which had a broad spectrum of activities in vasodilation and also in asthma, a compound for chemical optimisation was identified. The new compounds could be characterised with the already established models for vasodilation *in vitro* and *in vivo* (Table 5), and in 1989 the compound sildenafil acquired candidate status for clinical development as an anti-hypertensive (Campbell 2000).

Erectile dysfunction had been considered by most clinicians and by the consultants for industry as a trivial, “non-ethic” issue and a condition predominantly attributable to psychologic causes. On the contrary, the interest of treating impotence was high, the number of men suffering from this condition was enormous, and a variety of procedures were applied in an attempt to treat impotence in men including (1) injecting sheep testis extracts, (2) using under-pressure devices and (3) surgical procedures. During such a surgical operation, papaverine was used as intraoperative vasodilator

Table 5 Animal models available for studies of cardiovascular regulation and myocardial contractility in 1985

Animal model	Heart rate	Blood pressure	Coronary flow	Cardiac functions	Pulmonary artery pressure
Anaesthetised dog	x	x	x	x	x
Conscious dog	x	x		x	
Anaesthetised rat	x	x		x	(x)
Pithed rat	x	x		x	
Hypertensive, conscious rat	x	x			

Animal models and the techniques for measurement of hemodynamic parameters were established for the development of antihypertensive and cardiotoxic drugs. The efficacy of PDE3 and PDE5 inhibitors on cardiac contractility, coronary and pulmonary pressures and flows has been quantified in these models. Primary read-outs were measured after placing catheters into carotid, femoral or pulmonary artery and left cardiac ventricle and flow probes on peripheral and coronary arteries. Anaesthetised, instrumented animals were mechanically ventilated via endotracheal tubes and usually venous catheters were used for drug administration. Detailed descriptions of these methods are found in Vogel (2002).

and after injection into the corpus cavernosum an erection of the patient's penis was observed (Virag et al. 1981). The description of this accidental observation and the knowledge of Kukovetz's experiments stimulated GS Brindley to intensively study the effects of intracavernosal injection of papaverine and several other vasoactive drugs (Brindley 1982, 1986). The results of this engaged investigation was published in 1983 in an unusual, unique presentation (Klotz 2005).

Clinical success of sildenafil in treatment of coronary artery disease was limited due to the relatively short half-life of 4 h and limiting adverse effects such as "blue vision", flushing and headache at doses above 50 mg. Improved erectile function had been reported incidentally among men participating in the clinical trials of sildenafil conducted by Pfizer. These reports along with the realisation that PDE5 inhibitors such as sildenafil can amplify the naturally intended sexual functions was in accord with the reports by Ignarro that naturally derived NO released from nerves upon sexual stimulation or from exogenous NO donors increases cGMP synthesis and thereby works as a mediator of erection (Ignarro et al. 1990). It was 1992 when gears were changed for sildenafil development to focus on treatment of erectile dysfunction excellently described by Campbell (Campbell 2000) and a recent review (Ghofrani et al. 2006).

Pharmacological studies of PHT models had already shown that the relief of symptoms elicited by inhaled prostacyclin could be potentiated by PDE 3/4 and/or PDE5 inhibitors (Schemuly et al. 1999), and these medications could be transferred to clinical studies (Ghofrani et al. 2002a, b). Later, the NO-regulated hypoxic vasoconstriction in the lung, which protects against mismatch of ventilation and perfusion within the lung microcirculation, indicated that sildenafil alone may amplify the action of NO whose local release has been pathologically downregulated, thereby improving pulmonary microcirculation and ventilation/perfusion matching (V/Q ratio) (Grimminger et al. 1995). Finally, these results led to the successful introduction of sildenafil (RevatioTM) into PHT therapy. The same principle of selective demand-dependent regulation of microperfusion seems to be embodied by NO-mediated neuro-vascular coupling in the brain which has been investigated in animal models (Rosengarten et al. 2006). Application of the PDE5-selective inhibitors in treatment of a number of other maladies related to dysfunction in vascular and non-vascular systems continues to emerge.

6 New PDE Inhibitors Against Airway Diseases Without Vomiting

6.1 The First Goal: An Improved Bronchodilator with Anti-inflammatory Potential

The starting point of the new anti-asthma drug program in the company of the authors (originally Byk Gulden, later Altana and today Nycomed) was a running cardiovascular program with dihydro-pyridazinone compounds, which were chemical relatives

of the bipyridines such as aminone (Wetzel and Huel 1988). Having established the biochemical PDE assay, it turned out that these compounds were PDE inhibitors. In the already existing isolated organ models and in vivo models for bronchoconstriction, it was demonstrated that these compounds were more potent than theophylline and showed bronchodilatory activity in vitro and in vivo. Byk Gulden at that time marketed Euphylong – a long duration preparation of theophylline which could be dosed individually in order to avoid the well-known toxic effects of theophylline. This administration, however, was complicated and a development of a new medication could be a task for the young (and small) research team. Taking all arguments together a decision was drawn in the early 1980s to identify a new candidate substance for treatment of asthma and the new medication should be identified following certain (negative) criteria: it should (1) not be a xanthine, (2) not be an adenosine antagonist, (3) be less toxic than theophylline and (4) have a better therapeutic window than theophylline. If PDE inhibition was a relevant mechanism of theophylline, and many arguments were in favour of this assumption, then a more selective compound could be expected to have a better therapeutic window.

Asthma at that time was treated mainly with β_2 -agonists, oral and inhaled glucocorticoids and by cromoglycate. It was accepted that β_2 -agonists relaxed the bronchial muscle without an influence on inflammation whereas glucocorticoids reduced the chronic inflammatory process in the lung without influencing bronchoconstriction directly. Glucocorticoids – in particular the oral but also the inhaled forms – were met with scepticism. Since patients after oral steroid treatment suffered from loss of bone density and were at high risk for bone fractures, a so-called “steroidphoby” oppressed public opinion. Also, possible retardation of the growth of children who were taking steroids was discussed. This was a further argument to develop alternative anti-inflammatory agents to replace the “dangers” associated with use of the steroids. The available knowledge indicated that smooth muscle contraction as well as activation of inflammatory cells might be simultaneously reduced in the presence of PDE inhibitors.

6.2 *Animal Models for Asthma*

Animal disease models that somehow reflect the contemporary view about the pathomechanisms of a disease and for asthma are listed in Table 6. Since 1962 asthma was defined by the American Thoracic Society by (1) variable obstruction of airflow and (2) airway hyper-reactivity (American Thoracic Society 1962; Hargreave 1989). Obstruction of airways was attributed to a reduction of the airway lumen by bronchoconstriction which could be measured non-invasively. The mainstay model was the anaesthetised, mechanically ventilated guinea pig. The jugular vein, carotid artery and trachea were cannulated with catheters for drug and spasmogen administration, blood pressure monitoring and mechanical ventilation, respectively. During bronchospasms the airflow decreased while the airway pressure and respiratory rate increased dramatically, and, from these parameters,

Table 6 Animal models for studies of airway diseases

Pathophysiology	Pretreatment	Challenge	Read-out	PDE involved
Bronchoconstriction, anaesthetised or conscious	Healthy	His, Ach, PAF, LTD ₄	Protection vs. bronchoconstriction	3/4
Hyperreactivity	PAF, OVA-sensitisation	Ach, His, Adenosine	Dose/response curve, PC20	3/4, 4, 4
Allergic inflammation	OVA-sensitisation	OVA, Inhalative	Protection vs. bronchoconstriction, BAL, cell influx, eosinophilia	4
Systemic inflammation	Healthy	LPS iv	TNF α release, BAL, cell influx, neutrophilia	4
Pulmonary inflammation	Cigarette smoke, 3–11 days	Chronic	neutrophilia	4
Emphysema	Cigarette smoke, 6 months	Chronic	BAL cell influx, parenchymal destruction	
Pulmonary hypertension	MCT, 2–4 weeks; hypoxia, 2 weeks	Chronic	Pulmonary artery pressure, vascular remodelling	4, 5
Fibrosis	Bleomycin, 2 weeks	Chronic	Pulmonary artery pressure, vascular remodelling	4

The anaesthetised guinea pig was the mainstay model in 1980 for measuring airway mechanics translating to bronchoconstriction during the asthma attack. Transition to conscious animals and to quantification of pulmonary inflammation was developed when the mechanistic concept of airway hyper-reactivity and the underlying inflammation was penetrated in 1989. COPD models were established after 1990

changes in conductance and resistance of airways could be calculated. In fact, a prevention or protection against bronchoconstriction was measured assuming that this protection translates to improvement of FEV1 in asthma.

Anaesthesia had two clear disadvantages: (1) a depressant effect on respiration and (2) the necessity to dose by the parenteral route and not by oral administration. Therefore, new models with conscious animals needed to be established. With a non-invasive, thoracographic technique, (1) the change of respiratory rate, (2) the peak flow and (3) the time of onset of dyspnea were recorded (Kilian et al. 1989). The tool was a probe which was placed around the thorax of the animal (a mercury-in-rubber strain gauge) which by measuring movements of the thorax, was useful in detecting (1) breathing pattern, (2) breathing frequency and (3) coughs. More quantitative, but for routine purposes less suitable, methods were “whole-body” and “half-body” plethysmography with conscious animals mimicking the principle of body plethysmography in the clinics. These methods recorded specific airway resistance calculated from the phase shift between thorax and nasal flow curves. These methods and their variants are described in detail by Underwood et al. (1993).

An asthma attack in patients and in animal models was separated into an early (EAR) and late asthmatic reaction (LAR). EAR represented the dyspnoea induced by bronchoconstriction, which was related to the mediators histamine and LTC4 released from mast cells immediately after allergen contact. LAR started 4–6 h after the EAR, lasted for up to 24 h and was followed by hyper-reactivity of the airways (Cockroft 1983). Hyper-reactivity represented an exaggerated bronchoconstrictor response to a variety of stimuli such as histamine, adenosine, carbachol, exercise or cold air, which appeared to be linked with perennial asthma but apparently were not the consequence of acute contact with allergen. In 1983, J Morley had discovered that inhalation of platelet-activating factor (PAF) could induce hyper-reactivity in normal, non-sensitised guinea pigs. In the “pre-sensitisation” time period, this finding was the basis for the experimental option to assess hyper-reactivity and, in addition, in a conceptual way was the basis for the transient hypothesis that platelets might be important cellular players in mucosal inflammation and airway hyper-reactivity (Morley et al. 1984). Thus, PAF-induced bronchial hyper-reactivity of guinea pigs became an established method (Reaburn et al. 1994). Hyper-reactivity was tested by obtaining a dose–response curve for carbachol or histamine in the absence or presence of drugs and the shift of the dose–response curves to higher (less sensitive) values was given as PC20 defined as “provocative concentration to reduce FEV1 by 20%” (Lötvall et al. 1998; Hannon et al. 2001).

In parallel, aiming at the allergic, immunologic feature of the disease, a sensitisation procedure with OVA was developed and sensitised guinea pigs became the standard model for investigation of allergic mechanisms (Andersson 1980). In sensitised guinea pigs, it occurred that adenosine became a bronchoconstrictor agonist and evoked hyperreactivity versus other contracting agents which was in line with the exchanged role of adenosine in human asthma (Cushley and Holgate 1985). Thus, hyper-reactivity could now be established by adenosine treatment and the shift of PC20 for methacholine was experimentally accessible (Thorne and Broadley 1994).

Another model was the “SRS-A-model” recording the LTC₄-mediated contraction. Guinea pigs had to be pretreated with antihistamines and COX inhibitors in order to dissect out the constriction due to LTC₄. Furthermore, the isolated trachea from sensitised guinea pigs served for in vitro experiments which predicted the drug-sensitivity in the isolated organ model, and it was shown that the mucosa was responsible for the reactivity towards OVA (Goldie et al. 1986).

In 1988, the concept of asthma pathogenesis changed to the view that the disease persists outside the acute episodes and that a continuous pulmonary inflammation is the prerequisite of allergic attacks and hyperresponsiveness (Kay 1987; Barnes 1989; Hargreave 1989). Asthma was described as an “eosinophilic desquamative bronchitis” and mechanistically a cellular hierarchy emerged with (1) dendritic cells to recognise the antigens, (2) antigen-specific TH2 cells to release IL-5 and IL-4 and to orchestrate the inflammation and finally (3) terminal effector cells such as eosinophils, neutrophils and mast cells to release tissue-degrading enzymes, ROS, cytokines and mediators.

Consequently, following these conceptual papers pulmonary inflammation was measured in patients as well as in OVA-sensitised animal models by bronchoalveolar lavage (BAL). Sensitised animals were treated with substance and 1 h later allergen challenge was performed by inhalation. Lavages with buffer were performed at various time points after challenge – and complex analyses – a fixed time point of 24 h was chosen to demonstrate inflammatory changes as well as drug-related effects (Coyle et al. 1988; Schudt et al. 1991a, b, c; Underwood et al. 1993). The predominant use of guinea pigs had historical reasons since their allergic reactions were mediated by IgE and histamine as it occurs in human. Unfortunately, guinea pigs were highly susceptible to pneumonia and often blood and/or pulmonary eosinophilia were observed without any specific reason. Rats, however, were used for most other inflammatory models; pharmacokinetic and toxicological studies were routinely performed in rats; hence, the use of laboratory animals in respiratory research was shifted to rats (Hatzelmann et al. 1996b).

Chronic asthma was characterised by remodelling of airways, by thickening of the smooth muscle and the mucous layer which was due to increased cell mass and edema. In mice, these long-term changes could be mirrored in a chronic asthma model using OVA-sensitised mice exposed to aerosolised OVA at 3 days/week for 6 weeks. In the last 2 weeks, the drug was added daily and drug-induced changes of (1) subepithelial collagenisation, (2) thickening of the airways, (3) goblet cell hyperplasia and (4) TGFβ accumulation were reported (Kumar et al. 2003).

6.3 Zardaverine and Tolafentrine

Zardaverine was synthesised in 1984 and was determined to be a dual PDE inhibitor with equivalent potency for PDE3 and PDE4 which translated to the cells and isolated organs available at that time point (Tables 2 and 3). Bronchoconstriction in vivo was inhibited 100-fold more potently by zardaverine than by theophylline

but with similar efficacy. In sensitised OVA-challenged guinea pigs, zardaverine reduced allergic bronchoconstriction and reduced hyper-reactivity induced by Ach (Kilian et al. 1989). These data were confirmed in 1989 when inhibition of bronchial eosinophilia could be demonstrated in the freshly established guinea pig BAL model (Table 6) (Schudt et al. 1991a, b, c; Tenor and Schudt 1996). The risk for AEs was tested in rodent models using behavioural effects (Wachtel 1983) and for vomiting in dogs (Burkman 1982), and since emesis was found to be low in comparison with rolipram, the results opened the possibility to try both the oral and the inhalative administration routes. Pharmacologically, the efficacy of inhaled/instilled and oral zardaverine could be demonstrated in guinea pigs and the prerequisites for human studies were provided by oral and inhalation toxicology. Clinical trials with oral and iv administration started in 1988. Disappointingly, but not totally surprising, at oral doses that were efficacious for bronchodilation, AEs involving the central nervous system, and especially vomiting, were experienced which discouraged further development for oral administration. In inhalation studies, zardaverine was well tolerated. FEV1 increases of 200–400 ml – depending on the dose – were shown; however, the duration of dilation was not sufficient for a long-term protection. The interesting question as to whether the inflammatory process in the lungs of the asthmatic was influenced could be qualitatively assessed by studying EAR and LAR after provocation and considerable reductions of LAR were reported (Hatzelmann et al. 1996a). When zardaverine development was terminated in 1991 due to its fast elimination, 250 patients had received this drug by inhalation.

Benafentrine or AH 21-132 had been investigated at Sandoz and a bronchodilating efficacy was demonstrated in volunteers (Foster et al. 1992). Benafentrine was structurally related to papaverine and served as a lead structure for tolafentrine (Hatzelmann et al. 1996a). Also tolafentrine was a dual PDE3/4 inhibitor with bronchodilatory function after mediator or allergen stimulation, however, with ~20-fold higher potency (Beume et al. 1993; Schudt et al. 1993). In contrast to zardaverine, tolafentrine was scarcely adsorbed following oral administration. This characteristic would significantly reduce the expected AEs due to the swallowed fraction which was 40–50% of the inhaled material after inhalation therapy. The development of tolafentrine had successfully passed the inhalation studies in normal volunteers in phase I trials without complications. However, when the first four asthmatics received the puffs of inhalative tolafentrine, they experienced an unforeseen bronchoconstriction. The reason for this effect is not understood even today, but it led to termination of clinical development of tolafentrine for asthma.

6.4 Early Strategies for Avoiding Nausea and Vomiting

In the pioneering work of Wachtel, a Schering scientist, rolipram was characterised pharmacologically as a centrally active PDE4 inhibitor (Wachtel 1983). In rodents, rolipram reduced (1) body temperature, (2) locomotor activity, and (3) behavioural

effects, and in patients (4) an antidepressant activity had been demonstrated (Horowski and Sastre-Y-Hernandez 1985). The AEs such as nausea and vomiting prohibited any further development. Looking for the mode of action for cerebral efficacy, it was found that radioactive rolipram bound to brain structures in vivo with a dissociation constant which was <100-fold lower than the IC₅₀ for inhibition of catalytic activity. In binding studies with brain membranes, a dissociation constant of 1.2 nM was calculated and as binding site a membrane-bound PDE4 was identified. Thus, there was a high affinity and a low affinity rolipram binding site in the same enzyme (HARBS and LARBS), and the hypothesis was raised that PDE4 can switch between two conformations which was later corroborated by evidencing the coexistence of both conformations in one cloned and expressed enzyme (Torphy et al. 1992a, b).

These experiments indicated that emesis by PDE4 inhibitors might be related to HARBS. Similarly, with ROS production in neutrophils, we had observed that rolipram inhibition of release of ROS occurred with an IC₅₀ of 5 nM and was apparently mediated by HARBS (see Table 7). The IC₅₀ ratio between catalysis and ROS release was 42 for rolipram, 6 and 9 for zardaverine and denbufylline, but for the isochinolines papaverine, benafentrine and tolafentrine the ratio was <0.2. From these results, it was initially assumed that rolipram-mediated decrease in ROS release from neutrophils was mediated by its interaction with a PDE4 in the high affinity binding conformation. With the development of tolafentrine, it was determined that it did only weakly recognise the high affinity binding site of PDE4 and so differed from rolipram. The interpretation that tolafentrine did only weakly HARBS was supported by the fact that in clinical studies no emesis was observed with blood concentrations of up to 5 µM which exceeded the IC₅₀ >50-fold. Similar high blood concentrations (around 1 µM steady state plasma level after 80 mg infusion) were later achieved in a clinical study with PHT patients (Ghofrani et al. 2002b) showing that tolafentrine was the first example of a PDE4 inhibitor with low emetic potential due to avoidance of interaction with PDE4 in the high affinity conformation.

Table 7 Ratio between inhibition of PDE4 catalysis and ROS release in human neutrophils

Substance	IC ₅₀ (nmol/l)		
	PDE4 (PMN)	ROS (CL)	Ratio
Rolipram	210	5	42
Zardaverine	160	28	6
Denbufylline	100	11	9
Papaverine	110	530	0.21
Benafentrine	4,000	30,200	0.13
Tolafentrine	63	380	0.17

IC₅₀ values for inhibition of PDE4 catalytic activity were determined using enzyme from chromatographic fractions of a PMN homogenate containing mainly PDE4. IC₅₀ values for ROS release from neutrophils were calculated from concentration–response curves and chemoluminescence related to fMLP-induced oxidative burst was measured. Data are on file at Nycomed

A variety of animal species was investigated to study and predict the emetic potential in man. Neither (1) dog, nor (2) ferret has been reported to reveal a significant correlation between (1) blood concentrations for vomiting and (2) HARBS of PDE4 inhibition for a group of PDE4 inhibitors indicating that no reliable results were drawn from these models. Recently, with use of PDE4D knockout mice, the duration of alpha2-adrenoceptor-mediated anaesthesia was suggested as a correlate of emesis (Robichaud et al. 2002). This model may be relevant and sufficient, however, in the historical overview, it occurs that finally the surrogate for estimation of emetic potential was the IC50 ratio of (1) ROS release from neutrophils to (2) TNF release from MC. These assumptions guided the identification the next generation of PDE4 inhibitors which were synthesised on the basis of the rolipram structure (Christensen et al. 1996), and the most promising examples derived from this approach are piclamilast (Souness 1996), cilomilast (Barnette et al. 1998) and roflumilast (Hatzelmann et al. 2010).

6.5 *Animal Models for COPD*

Chronic bronchitis and emphysema as well as their clinical correlates, named the “pink puffer” and “blue bloater”, had been discussed since their definition (Filley 1967) and a variety of pathogenetic concepts had been put forward (Tudor and Voelkel 2002). When the increasing worldwide prevalence of COPD was published in 1997 (Murray and Lopez 1997), it appeared that this fast-growing disease had been neglected and a discussion about pathomechanisms and possible new strategies towards treatment of COPD started (Barnes 1998). COPD was recognised as a systemic disease with pathogenic changes in the small airways, characterised by (1) irreversible bronchial obstruction due to remodelling and hypersecretion, (2) mucociliary malfunction with exaggerated mucus production and inhibited transport as well as (3) reduced exercise tolerance (Barnes 1998). Smoking is the main environmental factor, but no information existed as to why about 15% of smokers were susceptible for the disease, whereas about 85% smokers were unaffected. COPD shows an “anomalous inflammatory process” in the lung with CD8+ and CD68+ cells in bronchial biopsies and macrophages and neutrophils in the induced sputum. Furthermore, enhanced serum albumin was found in BALs and TNF α was the major cytokine found in sputum. These inflammatory parameters could be imitated in an animal model which had initially been established for endotoxic shock (Fischer et al. 1993). LPS was given intravenously and 4 h later lungs were lavaged and cells and TNF α were quantified. This model in the BAL showed neutrophilia, increased macrophages, TNF α and serum albumin indicating the induced edema and was used as a screening model for pulmonary and systemic inflammation (Table 6). Corresponding cellular models for the characterisation of the various different pathophysiologies including (1) remodelling, (2) secretory and (3) vascular functions were established.

Cigarette smoke had been identified to be the relevant inducer of COPD in industrialised countries and therefore smoke-induced rodent systems were developed, which are compiled in a recent review (Churg et al. 2008). Since the rat is the most sensitive animal species for PDE4 toxicity, it was highly desirable to establish a smoke-induced rat model, but only a few laboratories succeeded (Lee et al. 2005). In cigarette smoke-induced mice or guinea pig models, however, it appeared that PDE4 inhibitors substantially reduced neutrophil influx into lungs whereas steroids were without effect (Fitzgerald et al. 2003, 2006). On the basis of these experiments, it was demonstrated that 3 days were sufficient to evoke the cell influx which is typical for COPD and an animal model of smoke-induced pulmonary inflammation was established (Weidenbach et al. 2008a), which was resistant to steroids and served as a routine model for identification of inhibitors of smoke-induced inflammation.

6.6 COPD Pathomechanisms Overlap with Oral PDE4 Inhibitor Potential

From the various disease mechanisms discussed for COPD (inflammation, mucociliary malfunction, architectural remodelling and oxidative stress, among others), COPD-related inflammation is mainly linked with (1) neutrophils, (2) T cells and (3) macrophages, yet structural cells are another source of inflammatory chemokines, cytokines, arachidonic acid metabolites or ROS. Mucociliary malfunction may largely be orchestrated by bronchial epithelial cells, yet modulated by numerous inflammatory mediators and tobacco smoke itself. Lung fibroblasts, airway and pulmonary vascular smooth muscle cells, lung endothelial and bronchial epithelial cells may account for architectural remodelling (Barnes and Rennard 2008).

All of these cells express PDE4, and in some cells (such as neutrophils or MCs), PDE4 is almost exclusively the cAMP-hydrolyzing PDE. As a corollary, PDE4 inhibitors modify a wealth of cellular functions, which may be relevant in COPD (summarised in Table 8). For example, in neutrophils PDE4 inhibitors potently diminish (1) release of superoxide radicals, (2) LTB₄ (Hatzelmann and Schudt 2001), (3) neutrophil elastase, (4) MMP9 (Jones et al. 2005), (5) the surface expression of CD11b (Sanz et al. 2007) or (6) trans-endothelial migration secondary to fMLP. In the same cell, PDE4 inhibitors reduce zymosan-induced (7) release of IL-8 (Au et al. 1998) that may act as an autocrine agent to facilitate neutrophil migration, comparable to LTB₄. In T cells, inhibitors of PDE4 diminish (1) proliferation, (2) formation of cytokines such as IFN γ or IL-2 (Giembycz et al. 1996; Hatzelmann and Schudt 2001) or (3) release of granzyme B from CD8+ T cells (Tenor et al. 2005), among others.

The bronchial epithelial cell is another target of PDE4 inhibitors (Dent et al. 1998; Fuhrmann et al. 1999; Mata et al. 2005). In vitro, exposure to tobacco smoke extract (1) reduces ciliary beat frequency (CBF) (Cohen et al. 2009; Simet et al. 2009),

Table 8 Isolated cell models for COPD in 2005

Model	Stimulus	Response	PDE
Neutrophils	fMLP	ROS, LTB ₄ , HNE, MMP9, CD11b, chemotaxis, adhesion	4
	Zymosan	IL-8	
Monocytes	LPS	TNF α , LTB ₄	4
Macrophages	LPS	TNF α , ROS, MMPs	1,3,4
CD8+ T-cells	AntiCD3/ (CD28)	Proliferation, IL2, IFN γ , Granzyme B	1,3,4
CD4+ T-cells	AntiCD3/ CD28	Proliferation, IL2, IFN γ	1,3,4
Bronchial epithelial cells	TNF	GM-CSF	1,4
	None	CFTR	
	None/TSE	Ciliary beat frequency	
	TSE/EGF	MUC5AC	
Lung fibroblasts	bFGF	Proliferation	1,3,4,5
	TGF β 1	Myofibroblast transition, CTGF, fibronectin, collagen I	
	Fibronectin/ TGF β	Chemotaxis, collagen gel contraction	
Airway smooth muscle cells	Serum	Proliferation	1,3,4,5
Pulmonary vascular smooth muscle cells	Serum	Proliferation, endothelin I	1,3,4,5
Endothelial cells	Thrombin	Hyperpermeability	1,2,3,4
	TNF α	E-selectin	

Enriched and purified cell populations have been prepared, which are being used for determination of PDE inhibitor efficacy in the various facets of COPD pathomechanisms. A multiple array of read-outs has been established for cells involved in inflammatory, mucociliary, vascular or proliferative functions. TSE is tobacco smoke extract

(2) impairs CFTR-driven Cl⁻ secretion (Kreindler et al. 2005; Cantin et al. 2006; Cohen et al. 2009) and (3) augments production of mucus proteins such as MUC5AC in bronchial epithelial cells altogether accounting for mucociliary malfunction in COPD (Takeyama et al. 2001). PDE4 inhibitors in turn (1) increase CBF and rescue from compromised CBF secondary to tobacco smoke (Cervin and Lindgren 1998; Wohlsen et al. 2006; Milara et al. 2008a), (2) augment CFTR activity (Barnes et al. 2005; Pedemonte and Galietta 2008) and (3) reduce tobacco smoke-induced MUC5AC expression (EJ Morcillo, personal communication). As a corollary from these findings, a PDE4 inhibitor may be expected to mitigate mucociliary malfunction in COPD.

PDE4 inhibitors further prevent the activation of human lung fibroblasts illustrated by a (1) reduction of proliferation, (2) myofibroblast transition, (3) excessive generation of extracellular matrix (fibronectin, collagen I), (4) chemotaxis, (5) collagen gel contraction (reflecting scar formation), release of cytokines such as (6) eotaxin or expression of (7) ICAM-1 cell adhesion molecule (Kohyama et al. 2002; Boero et al. 2006; Dunkern et al. 2007; Klar et al. 2007; Togo et al. 2009; Sabatini et al. 2010). Finally, PDE4 inhibitors attenuate formation of ROS in structural cells such as human airway epithelial cells, pulmonary vascular smooth

muscle cells, endothelial cells, aside from neutrophils mitigating the overall burden of oxidative stress (Nielson et al. 1990; Schudt et al. 1991b; Hatzelmann and Schudt 2001; Muzaffar et al. 2008; Milara et al. 2008b; Diebold et al. 2009).

The influence of PDE4 inhibitors on major disease mechanisms in COPD is further corroborated in animal models. For example, PDE4 inhibitors were shown to suppress (1) BAL neutrophil influx following short-term exposure of mice or guinea pigs to tobacco smoke (Fitzgerald et al. 2003; Leclerc et al. 2006; Weidenbach et al. 2008a,b), (2) the specific cellular pattern of lung adaptive and innate immunity (dendritic cells, B, CD4+, CD8+ T cells) evolving after long-term (7 months) exposure of mice to tobacco smoke (Martorana et al. 2008), (3) airspace enlargement and lung peripheral destruction following tobacco smoke exposure in mice over 7 months (Martorana et al. 2005), (4) bleomycin-induced lung fibrotic remodelling (Cortijo et al. 2009) and (5) pulmonary vascular remodelling and PHT secondary to monocrotalin or hypoxia in rats (Izikki et al. 2009).

The following additional aspects may further underpin the role of PDE4 inhibitors in COPD. In situ PDE4 activity is subject to a network of dynamic regulations at the transcriptional and post-translational (phosphorylation, protein–protein interaction) levels, and there is evidence to assume an augmented PDE4 activity in areas of inflammation or remodelling. In those areas, elevated levels of autocrine factors such as PGE2 or adenosine may be present that potentially strengthen cellular effects from a PDE4 inhibitor.

In summary, PDE4 inhibitors potentially restore normal physiology in inflammatory and structural cells that are imbalanced in COPD. In this respect, the profile of a PDE4 inhibitor apparently overlaps with pathomechanisms in COPD. Roflumilast, recently approved for severe COPD unfolds efficacy in all of the COPD-related *in vitro* (Table 8) and *in vivo* models. A summary of these preclinical effects has recently been published (Hatzelmann et al. 2010). The moderate but pleiotropic effects of the PDE4 inhibitor on a broad spectrum of cell functions that govern COPD may finally orchestrate the proven clinical efficacy of roflumilast to improve lung function and reduce exacerbation rates in this ailment.

The recent publication of four large-scale clinical trials demonstrating that the oral, once daily PDE4 inhibitor roflumilast improved lung function and the rate of acute exacerbations in patients afflicted from moderate to severe COPD (Calverley et al. 2009; Fabbri et al. 2009) may herald the age of PDE4 inhibitors as oral remedies in COPD. This, for the first time after more than two decades, adds a new and oral concept different from (inhaled) long-acting β_2 -adrenoceptor agonists (LABA), (inhaled) long-acting muscarinic receptor antagonists (LAMA), or inhaled corticosteroids (ICS) to the therapeutic armamentarium of physicians. Meanwhile, roflumilast received marketing authorization for severe COPD in several countries including EU/USA (Giembycz and Field 2010).

With roflumilast, a fully competitive PDE4 inhibitor for the PDE4 active site, has been developed, and it is effective and well tolerated in COPD (Fabbri et al. 2009; Calverley et al. 2009). The therapeutic potential of PDE4 inhibitors may have targets far beyond COPD to ailments that are comorbid (Barnes and Celli 2008) or to other ailments as diverse as asthma, lung and liver fibrosis, Alzheimer's, IBD,

acute myeloid leukaemia or B-cell lymphoma, osteoporosis, type II diabetes, depression, schizophrenia, psoriasis and atopic dermatitis to name a few where results from clinics or animal models have been provided. However, a higher degree of PDE4 inhibition than tolerable with the current concept of competitive PDE4 inhibitors may be required to address some of the above mentioned conditions with an oral PDE4 inhibitor. Only recently by researcher's at Decode (see chapter by M. E. Gurney et al., this volume) introduced allosteric PDE4D modulators, that dually dock to the catalytic centre and the UCR2 N-terminal region. Strikingly, opposite to rolipram, these allosteric PDE4D modulators showed a very favourable ratio between pharmacological efficacy in mouse models of cognition and a well-accepted correlate of emesis in this species (Burgin et al. 2010). The future will show whether allosteric PDE4 modulators may further advance the tale of PDE4 inhibitors as their next generation with an even improved efficacy and tolerability.

7 Summary and Outlook

In this historical overview we recall how serendipitous findings in the clinics discovered the therapeutical potential of natural compounds later identified as PDE inhibitors. The progress and stepwise understanding of the dimensions of the PDE superfamily, the isoenzymes tissue distribution, their function and inhibition with growing selectivity was redrawn. From the PDE1–5 families the history of PDE3, 4 and 5 was discussed in more detail since specific inhibitors have either already reached the shelf of the pharmacist or will be launched in near future. For PDE1 and PDE6 no specific inhibitors have been developed until now, and in case of PDE6 it does not seem prudent to interfere with the function of this enzyme family which is essential for perception of light by retinal photoreceptors. Development of PDE2 inhibitors for use in treatment of CNS maladies and sepsis are progressing and will be discussed in two subsequent chapters in this volume (see chapter by J. Surapisitchat and J. Beavo and the chapter by Y.Liu, et al.). Regarding PDE7–11, many ongoing research activities also in the pharmaceutical industry are noted but any therapeutic activity has to be proven in the future. PDE4 inhibition and its broad clinical potential justified a detailed description not only due to the author's personal engagement and dedication, but also because this development describes the inherent difficulties arising from obstinate adverse effects that needed to be discriminated by drug optimisation. The first quotation of Ro 20-1724 (Hamilton 1972) and Schering's rolipram (ZK 62-711), (Hedqvist et al. 1978) potent and selective PDE4 inhibitors, in the literature dates back to 1972 and 1978 and both compounds were in clinical studies. In 2010 once daily, oral roflumilast as the first PDE4 inhibitor received market authorisation. The path to the first PDE4 inhibitor was never simple; rather it was very difficult, required perseverance and a conviction of "*per aspera ad astra*". And "*asperae*" were plenty from the early emesis problems, the problems experienced due to the existence of

different PDE4 conformers down to the demanding identification of the COPD population that most profit from a PDE4 inhibitor.

For all mentioned PDE inhibitors, further improvements seem to be desirable and necessary. Relevant examples will be presented in several book chapters and finally, each successful development will represent one transient step on the path to the future.

References

- Allard E (1904) Über Theocoinvergiftung. *Arch Klin Med* 80:510–519
- Alousi AA, Farah AE, Leshar GY, Opalka CJ (1979) Cardiotoxic activity of amrinone-Win 40680 [5-amino-3, 4'-bipyridine-6(1H)-one]. *Circ Res* 45:666–677
- American Thoracic Society (1962) Definitions and classifications of chronic bronchitis, asthma and pulmonary emphysema. *Am Rev Respir Dis* 85:762–768
- Andersson P (1980) Antigen-induced bronchial anaphylaxis in actively sensitized guinea pigs. *Allergy* 35:65–71
- Au BT, Teixeira MM, Collins PD, Williams TJ (1998) Effect of PDE4 inhibitors on zymosan-induced IL-8 release from human neutrophils: synergism with prostanoids and salbutamol. *Br J Pharmacol* 123:1260–1266
- Barnes PJ (1998) New therapies for chronic obstructive pulmonary disease. *Thorax* 53:137–147
- Barnes PJ (2003a) Theophylline, new perspectives of an old drug. *Am J Respir Crit Care Med* 167:813–818
- Barnes PJ (2003b) New concepts in chronic obstructive pulmonary disease. *Annu Rev Med* 54:113–129
- Barnes PJ, Celli B (2008) Systemic manifestations and comorbidities of COPD. *Eur Respir J* 33:1665–1685
- Barnes PJ, Rennard SI (2008) Pathophysiology of COPD. In: Barnes PJ, Drazen J, Rennard SI, Thomson N (eds) *Asthma and COPD. Basic mechanisms and clinical management*. Academic, Amsterdam, pp 425–442
- Barnes AP, Livera G, Huang P, Sun C, O'Neal WK, Conti M, Stutts MJ, Milgram SL (2005) Phosphodiesterase 4D forms a cAMP diffusion barrier at the apical membrane of the airway epithelium. *J Biol Chem* 280:7997–8003
- Barnette MS, Grous M, Cieslinski LB, Burman M, Christensen SB, Torphy TJ (1995a) Inhibitors of phosphodiesterase IV (PDEIV) increase acid secretion in rabbit isolated gastric glands: Correlation between function and interaction with a high-affinity rolipram binding site. *J Pharmacol Exp Ther* 273:1396–1402
- Barnette MS, Manning CD, Cieslinski LB, Burman M, Christensen SB, Torphy TJ (1995b) The ability of phosphodiesterase IV inhibitors to suppress superoxide production in guinea pig eosinophils is correlated with inhibition of phosphodiesterase IV catalytic activity. *J Pharmacol Exp Ther* 273:674–679
- Barnette MS, Bartus JO, Burman M, Christensen SB, Cieslinski LB, Esser KM, Prabhakar US, Rush JA, Torphy TJ (1996) Association of the anti-inflammatory activity of phosphodiesterase 4 (PDE4) inhibitors with either inhibition of PDE4 catalytic activity or competition for [³H] rolipram binding. *Biochem Pharmacol* 51:949–956
- Barnette MS, Christensen SB, Essayan DM, Grous M, Prabhakar U, Rush JA, Kagey-Sobotka A, Torphy TS (1998) SB 207499 (Ariflo), a potent and selective second-generation phosphodiesterase 4 inhibitor: in vitro anti-inflammatory actions. *J Pharmacol Exp Ther* 284:420–426
- Beavo JA (1988) Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv Sec Messengers Phosphoprotein Res* 22:1–38

- Beavo JA, Conti M, Heaslip RJ (1994) Multiple cyclic nucleotide phosphodiesterases. *Mol Pharmacol* 46:399–405
- Benotti JR, Grossman W, Braunwald E, Davolos DD, Alousi A (1978) Hemodynamic assessment of amrinone. *N Engl J Med* 299:1373–1377
- Beume R, Kilian U, Brand U, Häfner D, Eltze M, Flockner D (1993) The bronchospasmolytic effects of the PDEIII/IV inhibitors B9004-70 and zardaverine – dependency on the route of administration in guinea pigs. *Am Rev Respir Dis* 174:A184
- Boero S, Silvestri M, Sabatini F, Nachira A, Rossi GA (2006) Inhibition of human lung fibroblast functions by roflumilast N-oxide. *Eur J Respir (Suppl)* 662s:P3845, abstract
- Boolell M, Allen MJ, Ballard SA, Gepi-Attee S, Muirhead GJ, Naylor AM, Osterloh IH, Gingell C (1996) Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int J Impot Res* 8:47–52
- Brindley GS (1982) Intracavernous injection of papaverine for erectile failure. *Lancet* 2:938
- Brindley GS (1986) Pilot experiments on the actions of drugs injected into the human corpus cavernosum penis. *Br J Pharmacol* 87:495–500
- Bundschuh DS, Eltze M, Barsig J, Wollin L, Hatzelmann A, Beume R (2001) In vivo efficacy in airway disease models of roflumilast, a novel orally active PDE4 inhibitor. *J Pharmacol Exp Ther* 297:280–290
- Burgin AB, Magnusson OT, Singh J, Witte P, Staker BL, Bjornsson JM, Thorsteinsdottir M, Hrafnisdottir S, Hagen T, Kiselyov AS, Stewart LJ, Gurney ME (2010) Design of phosphodiesterase 4D (PDE4D) allosteric modulators for enhancing cognition with improved safety. *Nat Biotechnol* 28:63–70
- Burkman AM (1982) Assessment of emetic and antiemetic activity. *J Pharmacol Meth* 8:165–171
- Butcher RW (1984) Phosphodiesterase after 20 years: an Introduction. *Adv Cycl Nucl Protein Phos Res* 16:1–12
- Calverley PM, Rabe KF, Goehring UM, Kristiansen S, Fabbri LM, Martinez FJ, M2–124 and M2–125 study groups (2009) Roflumilast in symptomatic chronic obstructive pulmonary disease. Two randomized clinical trials. *Lancet* 374:685–94
- Campbell SF (2000) Science, art and drug discovery: a personal perspective. *Clin Sci* 99:255–260
- Cantin AM, Hanrahan JW, Bilodeau G, Ellis L, Dupuis A, Liao J, Zielenski J, Durie P (2006) Cystic fibrosis transmembrane conductance regulator function is suppressed in cigarette smokers. *Am J Respir Crit Care Med* 173:1139–44
- Cervin A, Lindgren S (1998) The effect of selective phosphodiesterase inhibitors on mucociliary activity in the upper and lower airways in vitro. *Auris Nasus Larynx* 25:269–76
- Chikanza IC, Jawed SJ, Blake DR, Perrot S, Menkes CI, Barnes CG, Perry JD, Wright MG (1996) Treatment of patients with rheumatoid arthritis with RP73401 phosphodiesterase type IV inhibitor. *Arthritis Rheum* 39:282
- Christensen SB, DeWolf WE, Ryan MD, Torphy TJ (1996) Molecular aspects of inhibitor interaction with PDE4. In: Schudt C, Dent G, Rabe KF (eds) *Phosphodiesterase inhibitors*. Academic, London
- Churg A, Cosio M, Wright JL (2008) Mechanism of cigarette smoke-induced COPD: insights from animal models. *Am J Physiol Lung Cell Mol* 294:L612–L631
- Cockcroft DW (1983) Mechanism of perennial allergic asthma. *Lancet* 322:253–256
- Cohen NA, Zhang S, Sharp DB, Tamashiro E, Chen B, Sorscher EJ, Woodworth BA (2009) Cigarette smoke condensate inhibits transepithelial chloride transport and ciliary beat frequency. *Laryngoscope* 119:2269–2274
- Compton CH, Gubb J, Nieman R, Edelson J, Ohad A, Bakst A, Ayres JG, Creemers JP, Schultze-Werninghaus G, Brambilla C, Barnes NC (2001) Cilomilast, a selective phosphodiesterase-4 inhibitor for treatment of patients with chronic obstructive pulmonary disease: a randomised, dose-ranging study. *Lancet* 358:265–270
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511

- Cortijo J, Bou J, Cardelus I, Llenas J, Morcillo E, Gristwood RW (1993) Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus. *Br J Pharmacol* 108:562–568
- Cortijo J, Iranzo A, Milara X, Mata M, Cerdá-Nicolás M, Ruiz-Saurí A, Tenor H, Hatzelmann A, Morcillo EJ (2009) Roflumilast, a phosphodiesterase 4 inhibitor, alleviates bleomycin-induced lung injury. *Br J Pharmacol* 156:534–544
- Coyle AJ, Urwin SJ, Page CP, Touvay C, Villain B, Braquet P (1988) The effect of selective PAF antagonist BN52021 on PAF- and antigen-induced bronchial hyper-reactivity and eosinophil accumulation. *Eur J Pharmacol* 148:51–58
- Cushley MJ, Holgate ST (1985) Adenosine-induced bronchoconstriction in asthma: role of mast cell-mediator release. *J Allergy Clin Immunol* 75:272–278
- Dent G, Rabe KF, Giembycz MA (1990) Inhibition of eosinophil respiratory burst activity by type IV- but not by type III-selective phosphodiesterase inhibitors. *A Rev Respir Dis* 141:A878
- Dent G, Giembycz MA, Rabe KF, Barnes PJ (1991) Inhibition of eosinophil cyclic nucleotide PDE activity and opsonin-stimulated respiratory burst by “type IV”-selective PDE inhibitors. *Br J Pharmacol* 103:1339–1346
- Dent G, White SR, Tenor H, Bodtke K, Schudt C, Leff AR, Magnussen H, Rabe KF (1998) Cyclic nucleotide phosphodiesterase in human bronchial epithelial cells: characterization of isoenzymes and functional effects of PDE inhibitors. *Pulm Pharmacol Ther* 11:47–56
- Diebold I, Djordjevic T, Petry A, Hatzelmann A, Tenor H, Hess J, Görlach A (2009) Phosphodiesterase 2 mediates redox-sensitive endothelial cell proliferation and angiogenesis by thrombin via Rac1 and NADPH oxidase 2. *Circ Res* 104:1169–77
- Dunkern T, Hatzelmann A (2005) The effect of sildenafil on human platelet secretory function is controlled by a complex interplay between phosphodiesterases 2, 3 and 5. *Cell Signal* 17:331–339
- Dunkern TR, Feurstein D, Rossi GA, Sabatini F, Hatzelmann A (2007) Inhibition of TGF- β induced lung fibroblast to myofibroblast conversion by phosphodiesterase inhibiting drugs and activators of soluble guanylyl cyclase. *Eur J Pharmacol* 572:12–22
- Engelstaetter R, Wingertzahn M, Schmid-Wirlitsch C, Bredenbröcker D, Leichtl S, Wurst W (2005) Roflumilast, an investigational, oral, once daily phosphodiesterase 4 inhibitor, did not exhibit acute bronchodilatory activity after a single dose. *Ann Allergy Asthma Immunol* 94:169
- England PJ, Shahid M (1987) Effects of forskolin on contractile responses and protein phosphorylation in the isolated perfused heart. *Br J Pharmacol* 264:687–695
- Fabbri LM, Calverley PM, Izquierdo-Alonso JL, Bundschuh DS, Brose M, Martinez FJ, Rabe KF (2009) M2-127 and M2-128 study groups. Roflumilast in moderate-to-severe chronic obstructive pulmonary disease treated with long acting bronchodilators: two randomised clinical trials. *Lancet* 374:695–703
- Farah AE, Alousi AA (1978) New cardiotoxic agents: a search for digitalis substitute. *Life sci* 22:1139–1148
- Filley GF (1967) Emphysema and chronic bronchitis: clinical manifestations and their physiologic significance. *Med Clin North Am* 51:283–292
- Fischer E, Ach L (1895) Synthese des Cafféins. *Berichte der deutschen Gesellschaft* 28:3135
- Fischer W, Schudt C, Wendel A (1993) Protection by phosphodiesterase inhibitors against endotoxin-induced liver injury in galactosamine-sensitized mice. *Biochem Pharmacol* 12:2399–2404
- Fitzgerald MF, Spicer D, Henning R (2003) Efficacy of the PDE4 inhibitor Bay 19-8004 and a steroid in tobacco smoke-induced models of pulmonary inflammation in the mouse. *Am J Respir Crit Care Med* 167:A91
- Fitzgerald MF, Spicer D, McAulay AE, Wollin L, Beume R (2006) Roflumilast but not methylprednisolone inhibited cigarette smoke-induced pulmonary inflammation in guinea pigs. *Eur Respir J Suppl* 663s:P3850, abstract
- Foster RW, Rakshi K, Carpenter JR, Small RC (1992) Trials of the bronchodilator activity of the isoenzyme-selective phosphodiesterase inhibitor AH 21-132 in healthy volunteers during methacholine challenge test. *Br J Clin Pharmacol* 34:527–534

- Francis SH, Lincoln TM, Corbin JD (1980) Characterization of a novel cGMP binding protein from rat lung. *J Biol Chem* 255:620–626
- Frossard N, Landry Y, Pauli G, Ruckstuhl M (1981) Effects of cyclic AMP- and cyclic GMP-phosphodiesterase inhibitors on immunological release of histamine and lung contraction. *Br J Pharmacol* 73:933–938
- Fuhrmann M, Jahn HU, Seybold J, Neurohr C, Barnes PJ, Hippenstiel S, Kraemer HJ, Suttrop N (1999) Identification and function of cyclic nucleotide phosphodiesterase isoenzymes in airway epithelial cells. *Am J Respir Cell Mol Biol* 20:292–302
- Gantner F, Kupferschmidt R, Schudt C, Wendel A, Hatzelmann A (1997a) In vitro differentiation of human monocytes to macrophages: change of PDE profile and its relationship to suppression of tumour necrosis factor- α release by PDE inhibitors. *Br J Pharmacol* 121:221–231
- Gantner F, Tenor H, Gekeler V, Schudt C, Wendel A, Hatzelmann A (1997b) Phosphodiesterase profiles of highly purified human peripheral blood leukocyte populations from normal and atopic individuals: a comparative study. *J Allergy Clin Immunol* 100:527–537
- Gantner F, Schudt C, Wendel A, Hatzelmann A (1999) Characterization of the phosphodiesterase (PDE) pattern of in vitro-generated human dendritic cells (DC) and the influence of PDE inhibitors on DC functions. *Pulm Pharmacol Ther* 12:377–386
- Ghofrani HA, Wiedemann R, Rose F, Olschewski H, Schermuly RT, Weissmann N, Seeger W, Grimminger F (2002a) Combination therapy with oral sildenafil and inhaled iloprost for severe pulmonary hypertension. *Ann Intern Med* 136:515–522
- Ghofrani HA, Rose F, Schermuly RT, Olschewski H, Wiedemann R, Weissmann N, Schudt C, Tenor H, Seeger W, Grimminger F (2002b) Amplification of the pulmonary vasodilatory response to inhaled iloprost by subthreshold phosphodiesterase types 3 and 4 inhibition in severe pulmonary hypertension. *Crit Care Med* 30:2489–2492
- Ghofrani HA, Osterloh IH, Grimminger F (2006) Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. *Nature Rev* 5:689–702
- Giembycz MA, Field KF (2010) Roflumilast: first phosphodiesterase 4 inhibitor approved for treatment of COPD. *Drug Des Dev Ther* 4:147–158
- Giembycz MA, Corrigan CJ, Seybold J, Newton R, Barnes PF (1996) Identification of cyclic AMP phosphodiesterases 3, 4 and 7 in human CD4+ and CD8+ T- lymphocytes: role in regulating proliferation and the biosynthesis of interleukin-2. *Br J Pharmacol* 118:1945–1958
- Goldie RG, Papadimitriou JM, Paterson JW, Rigby PJ, Self HM, Spina D (1986) Influence of the epithelium on responsiveness of guinea-pig isolated trachea to contractile and relaxant agonists. *Br J Pharmacol* 87:5–14
- Greene JA, Paul WD (1937) The action of theophylline with ethylenediamine on intrathecal and venous pressure in cardiac failure and on bronchial obstruction in cardiac failure and bronchial asthma. *J Am Med Assoc* 109:1712
- Grimminger F, Spriesterbach R, Weissmann N, Walmrath D, Seeger W (1995) Nitric oxide generation and hypoxic vasoconstriction in buffer-perfused rabbit lungs. *J Appl Physiol* 78:1509–1515
- Grootendorst DC, Gauw SA, Baan R, Kelly J, Murdoch RD, Sterk PJ, Rabe KF (2003) Does a single dose of the phosphodiesterase 4 inhibitor, cilomilast (15 mg), induce bronchodilation in patients with chronic obstructive pulmonary disease? *Pulm Pharmacol Ther* 16:115–129
- Grueter R (1910) Über leicht lösliche Verbindungen des Theophyllins. *Therapeutische Monatshefte* 24:613
- Hamilton TC (1972) The effects of some phosphodiesterase inhibitors on the conductance of the perfused vascular beds of the chloralosed cat. *Br J Pharmacol* 46:386–394
- Hannon JP, Tigani B, Williams I, Mazzoni L, Fozard JR (2001) Mechanism of airway hyperresponsiveness to adenosine induced by allergen challenge in actively sensitized Brown Norway rats. *Br J Pharmacol* 132:1509–1523
- Hargreave FE (1989) Late-phase asthmatic responses and airway inflammation. *J Allergy Clin Immunol* 83:525–527

- Harris AL, Conell MJ, Ferguson EW, Wallace AM, Gordon RJ, Pagani ED, Silver PJ (1989) Role of low Km cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea pig. *J Pharmacol Exp Ther* 251:199–206
- Hatzelmann A, Schudt C (2001) Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *J Pharmacol Exp Ther* 297:267–279
- Hatzelmann A, Tenor H, Schudt C (1995) Differential effects of non-selective and selective phosphodiesterase inhibitors on human eosinophil functions. *Br J Pharmacol* 114:821–831
- Hatzelmann A, Engelstätter R, Morley J, Mazzoni L (1996a) Enzymatic and functional aspects of dual-selective PDE3/4 inhibitors. In: Schudt C, Dent G, Rabe KF (eds) *Phosphodiesterase inhibitors*. Academic, London
- Hatzelmann A, Haefner D, Beume R, Schudt C (1996b) Automatic leukocyte differentiation in bronchoalveolar lavage fluid of guinea pigs and Brown-Norway rats. *J Pharmacol Toxicol Meth* 35:91–99
- Hatzelmann A, Morcillo E, Lungarella G, Adnot S, Sanjar S, Beume R, Schudt C, Tenor H (2010) The preclinical pharmacology of roflumilast – a selective, oral phosphodiesterase inhibitor in development for chronic obstructive pulmonary disease. *Pulm Pharmacol Ther* 23(4):235–256
- Hedqvist P, Fredholm BB, Olundh S (1978) Antagonistic effects of theophylline and adenosine on adrenergic neuroeffector transmission in the rabbit kidney. *Circ Res* 43:592–598
- Hermann GR, Aynesworth MB, Martin J (1937) Successful treatment of persistent extreme dyspnea, “status asthmaticus” – use of theophylline ethylene diamine (aminophylline, U.S.P.) intravenously. *J Lab Clin Med* 23:135
- Hidaka H, Asano T (1976) Human platelet 3':5'-cyclic nucleotide phosphodiesterase. *Biochem Biophys Acta* 429:485–497
- Hirsch S (1922) Klinischer und experimenteller Beitrag zur krampflosenden Wirkung der Purinderivate. *Klinische Wochenschrift* 1:615–618
- Holden CA, Chan SC, Hanifin JM (1986) Monocyte localization of elevated cAMP phosphodiesterase activity in atopic dermatitis. *J Invest Dermatol* 87:372–376
- Honerjäger P, Schäfer-Korting M, Reiter M (1981) Involvement of cyclic AMP in direct inotropic action of amrinone. *Naunyn-Schmiedeberg's Arch Pharmacol* 318:112–120
- Horowski R, Sastre-Y-Hernandez M (1985) Clinical effects of the neurotropic selective cAMP phosphodiesterase inhibitor rolipram in depressed patients: global evaluation of the preliminary reports. *Curr Ther Res* 38:23–29
- Ignarro LJ, Bush PA, Buga GM, Wood KS, Fukuto JM, Rajfer J (1990) Nitric oxide and cyclic GMP formation upon a electric fields stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem Biophys Res Comm* 170:843–850
- Izikki M, Raffestin B, Klar J, Hatzelmann J, Marx D, Tenor H, Zadigue P, Adnot S, Eddahibi S (2009) Effects of roflumilast, a phosphodiesterase-4 inhibitor, on hypoxia- and monocrotaline-induced pulmonary hypertension in rats. *J Pharmacol Exp Ther* 330:54–62
- Jones NA, Boswell-Smith V, Lever R, Page CP (2005) The effect of selective phosphodiesterase isoenzyme inhibition on neutrophil function in vitro. *Pulm Pharmacol Ther* 18:93–101
- Kaliner M, Orange R, Austen F (1972) Immunological release of histamine and slow reacting substance of anaphylaxis from human lung. *J Exp Med* 136:556–566
- Kammer GM (1988) The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. *Immunol Today* 9:222–229
- Katsuki S, Arnold W, Mittal C, Murad F (1977) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J Cycl Nucl Res* 3:23–35
- Kay AB (1987) Inflammatory cells in acute and chronic asthma. *Am Rev Respir Dis* 135:S63–66
- Kilian U, Beume EM, Schudt C (1989) Is phosphodiesterase inhibition a relevant bronchospasmolytic principle? *Agents Actions (Suppl)* 28:331–348
- Klar J, Sabatini F, Schatton E, Burgbacher B, Rossi GA, Hatzelmann A, Tenor H (2007) Roflumilast N-oxide reduces human fibroblast function. *Eur Respir J (Suppl)* 544s:3258, abstract

- Kleine-Tebbe J, Wicht L, Gagne H, Friese A, Schunack W, Schudt C, Kunkel G (1992) Inhibition of IgE-mediated histamine release from human peripheral leukocytes by selective phosphodiesterase inhibitors. *Agents Actions* 36:200–206
- Klotz L (2005) How (not) to communicate scientific information: a memoir of the famous Brindley lecture. *Br J Urol* 95:956–957
- Knispel HH, Goessel C, Beckmann R (1992) Nitric oxide mediate relaxation in rabbit and human corpus cavernosum smooth muscle. *Urol Res* 20:253–257
- Kohyama T, Liu X, Wen FQ, Zhu YK, Wang H, Kim HJ, Takizawa H, Cieslinski LB, Barnette MS, Rennard SI (2002) PDE4 inhibitors attenuate fibroblast chemotaxis and contraction of native collagen gels. *Am J Respir Cell Mol Biol* 26:694–701
- Kreindler JL, Jackson AD, Kemp PA, Bridges RJ, Danahay H (2005) Inhibition of chloride secretion in human bronchial epithelial cells by cigarette smoke extract. *Am J Physiol Lung Cell Mol Physiol* 288:L894–902
- Kukovetz WR, Holzmann S, Wurm A, Pösch G (1979) Evidence for cGMP-mediated relaxant effects of nitro-compounds in coronary smooth muscle. *Naunyn-Schmiedeberg's Arch Pharmacol* 310:129–138
- Kumar M, Bhattacharya V (2007) Cilostazol: a new drug in the treatment intermittent claudication. *Recent Pat Cardiovasc Drug Discov* 2:181–185
- Kumar RK, Herbert C, Thomas PS, Wollin L, Beume R, Yang M, Webb DC, Foster PS (2003) Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. *J Pharmacol Exp Ther* 307:349–355
- Lad PM, Goldberg BJ, Smiley PA, Olson CV (1985) Receptor-specific threshold effects of cyclic AMP are involved in the regulation of enzyme release and superoxide production from human neutrophils. *Biochem Biophys Acta* 846:286–295
- Langendorff O (1895) Untersuchungen am überlebenden Säugethierherzen. *Arch ges Physiol* 61:291–332
- Leclerc O, Lagente V, Planquois JM, Bethelier C, Artola M, Eichholtz T, Bertrand CP, Schmidlin F (2006) Involvement of MMP-12 and phosphodiesterase type 4 in cigarette smoke-induced inflammation in mice. *Eur Respir J* 27:1102–1109
- Lee J-H, Lee D-S, Kim E-K, Oh Y-M, Shim T-S, Kim S-E, Lee Y-S, Lee S-D (2005) Simvastatin inhibits cigarette smoking-induced ephysema and pulmonary hypertension in rat lungs. *Am J Respir Crit Care Med* 172:987–983
- Leeman M, Lejeune P, Melot C, Naelje R (1987) Reduction in pulmonary hypertension and in airway resistances by enoximone (NDL 17, 043) in decompensated COPD. *Chest* 91:662
- LeJemtel TH, Keung E, Sonnenblick EH, Ribner HS, Matsumoto M, Davis R, Schwartz W, Alousi AA, Davolos D (1979) Amrinone: a new non-glycosidic, non-adrenergic cardiotonic agent effective in the treatment of intractable myocardial failure in man. *Circulation* 59:1098–1104
- Liechtenstein ML, Margolis S (1968) Histamine release in vitro: inhibition by catecholamines and methylxanthines. *Science* 161:982–983
- Lorenz KL, Wells JN (1982) Potentiation of the effects of sodium nitroprusside and of isoproterenol by selective phosphodiesterase inhibitors. *Mol Pharmacol* 23:424–430
- Lötvall J, Inman M, O'Byrne P (1998) Measurements of airway hyperresponsiveness: new considerations. *Thorax* 5:419–424
- Macht DI, Ting GC (1921) A study of antispasmodic drugs in the bronchus. *J Pharmacol Exp Ther* 18:373–398
- MacPhee CH, Harrison SA, Beavo JA (1986) Immunological identification of the major platelet low- K_m cAMP phosphodiesterase: probable target for anti-thrombotic agents. *Proc Natl Acad Sci* 83:6660–6663
- Magnus R (1904) Versuche am überlebenden Dünndarm von Säugethieren. *Pflüger's Arch* 102:123–151
- Marone G, Columbo M, Triggiani M, Cirillo R, Genovese A, Formisano S (1987) Inhibition of IgE-mediated release of histamine and peptide leukotriene from human basophils and mast cells by forskolin. *Biochem Pharmacol* 36:13–20

- Martorana PA, Beume R, Lucattelli M, Wollin L, Lungarella G (2005) Roflumilast fully prevents emphysema in mice chronically exposed to cigarette smoke. *Am J Respir Crit Care Med* 172:848–853
- Martorana PA, Lunghi B, Lucattelli M, de Cunto G, Beume R, Lungarella G (2008) Effect of roflumilast on inflammatory cells in lungs of cigarette smoke-exposed mice. *BMC Pulm Med* 8:17–26
- Mata M, Sarriá B, Buenestado A, Cortijo J, Cerdá M, Morcillo EJ (2005) Phosphodiesterase 4 inhibition decreases MUC5AC expression induced by epidermal growth factor in human airway epithelial cells. *Thorax* 60:144–52
- Maurice DH, Haslam RJ (1990) Molecular basis of synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol Pharmacol* 37:671–681
- May CD (1974) History of the introduction of theophylline into the treatment of asthma. *Clin Allergy* 4:211–217
- Milara J, Cortijo J, Armengot M, Banuls P, Gabarda E, Morcillo E (2008a) Effect of roflumilast, a PDE4 inhibitor on ciliary beat frequency in human nasal epithelial cells. *Eur Respir J Suppl* 639s:P3651
- Milara J, Cortijo J, Mata M, Donet E, Mauricio M, Morcillo EJ (2008b) The PDE4 inhibitor roflumilast N-oxide partly reversed TGFβ1-induced changes in collagen I and E-cadherin expression in human airway epithelial cells. *Eur Respir J (Suppl)* 639s:P3648, abstract
- Morley J, Sanjar S, Page CP (1984) Platelet activation as a basis for asthma exacerbation. *Lancet* 2:1142–1144
- Murray JL, Lopez AD (1997) Alternative projections of mortality and disability by cause 1990–2020: global burden of disease study. *Lancet* 349:1498–1504
- Muzaffar S, Shukla N, Angelini GD, Jeremy JY (2008). Roflumilast N-oxide inhibits NADPH oxidase expression and activity in human pulmonary artery smooth muscle cells; *Proc Br Pharmacol Soc* 027P, (abstract).
- Naline E, Qian Y, Advenier C, Raeburn D, Karlsson J-A (1996) Effects of RP 73401, a novel, potent and selective phosphodiesterase type 4 inhibitor, on contractility of human, isolated bronchial muscle. *Br J Pharmacol* 118:1939–1944
- Nicholson CD, Jackman SA, Wilke R (1989) The ability of denbufylline to inhibit cyclic nucleotide phosphodiesterase and its affinity for adenosine receptors and the re-uptake site. *Br J Pharmacol* 97:889–897
- Nicholson CD, Challiss RAJ, Shahid M (1991) Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol Sci* 12:19–27
- Nielson CP, Vestal RE, RJ S, Heaslip R (1990) Effects of selective phosphodiesterase inhibitors on the polymorphonuclear leukocyte respiratory burst. *J Allergy Clin Immunol* 86:801–808
- O’Connell M, Dierdorf D, Greb WH, Mayer M-ER, Wolf D (1988) Efficacy of denbufylline in patients with multi-infarct dementia. *Dru Dev Res* 14:195–198
- Ohta K, Fukuchi Y, Grouse L, Mizutani R, Rabe KF, Rennard SI, Zhong N-S (2004) A prospective clinical study of theophylline safety in 3810 elderly with asthma or COPD. *Respir Med* 98:1016–1024
- Orange RO, Austen WG, Austen KF (1971) Immunological release of histamine and slow-reacting substance of anaphylaxis from human lung. *J Exp Med* 134:136–148
- Packer M, Carver JR, Rodeheffer RJ, Ivanhoe RJ, DiBianco R, Zeldis SM, Hendrix GH, Bommer WJ, Elkayam U, Kukin ML, Mallis GI, Sollano JA, Shannon J, Tendon PK, DeMets DL (1991) Effect of oral milrinone on mortality in severe heart failure. *N Engl J Med* 3325:1468–1475
- Pedemonte N, Galiotta L (2008). Stimulation of CFTR-dependent chloride secretion by roflumilast. *Eur Respir J Suppl* 644s:P3672.
- Persson CG (1985) On the medical history of xanthines and other remedies for asthma: a tribute to HH Salter. *Thorax* 40:881–886

- Pilcher JD (1912) The action of caffeine on the mammalian heart. *J Pharmacol Exp Med* 3:609–624
- Plant OH (1914) A note of the efficiency of the Knowlton-Starling isolated heart-lung preparation for testing the action of drugs. *J Pharmacol Exp Med* 5:603–614
- Poehc G, Kukovetz WR (1971) Papaverine – induced inhibition of phosphodiesterase activity in various mammalian tissues. *Life sci* 10:133–144
- Polson JB, Krzanowski JJ, Szentivani A (1982) Inhibition of a high cyclic AMP phosphodiesterase and relaxation of canine tacheal smooth muscle. *Biochem Pharmacol* 21:3403–3406
- Rabe KF, Tenor H, Dent G, Schudt C, Liebig S, Magnussen (1993) Phosphodiesterase isozymes modulating inherent tone in human airways: identification and characterization. *Am J Physiol* 264:L458–L464
- Rabe KF, Tenor H, Dent G, Schudt C, Nakashima M, Magnussen H (1994) Identification of PDE isoenzymes in human pulmonary artery and effect of selective PDE inhibitors. *Am J Physiol* 266:L536–L543
- Rall TW, Sutherland EW (1958) Formation of a cyclic adenine ribonucleotide by tissue particles. *J Biol Chem* 232:1065–1091
- Rall TW, West TC (1963) The potentiation of cardiac inotropic responses to norepinephrine by theophylline. *J Pharmacol Exp Ther* 139:269–274
- Rau S (2001) Von Coffein zum Furosemid. Entdeckung, Erforschung und Entwicklung der Diuretika im 20. Jahrhundert. Peter Lang, Frankfurt aM
- Reaburn D, Underwood SL, Lewis SA, Woodman VR, Battram CH, Tomkinson A, Sharma S, Jordan R, Souness JE, Webber SE, Karlsson J-A (1994) Anti-inflammatory and bronchodilator properties of RP 73401, a novel and selective phosphodiesterase type IV inhibitor. *Br J Pharmacol* 113:1423–1431
- Reeves ML, Leigh PK, England PJ (1987) The identification of a new cyclic nucleotide phosphodiesterase in human and guinea-pig cardiac ventricle. *Biochem J* 241:535–541
- Robichaud A, Savoie C, Stamatiou LN, Jolicoeur P, Rasori R, Chan CC (2002) Assessing the potential of PDE4 inhibitors in rats. *Br J Pharmacol* 135:113–118
- Robicsek SA, Krzanowski JJ, Szentivani A, Polson JB (1989) High pressure liquid chromatography of cyclic nucleotide phosphodiesterase from purified human T-lymphocytes. *Biochem Biophys Res Comm* 163:554–560
- Robicsek SA, Blanchard DK, Djeu JY, Krzanowski JJ, Szentivani A, Polson JB (1991) Multiple high-affinity cAMP-phosphodiesterases in human T-lymphocytes. *Biochem Pharmacol* 42:869–877
- Rosengarten B, Schermuly RT, Voswinkel R, Kohstall MG, Olschewski H, Weissmann N, Seeger W, Kaps M, Grimminger F, Ghofrani HA (2006) Sildenafil improves dynamic vascular function in the brain: studies in patients with pulmonary hypertension. *Cerebrovasc Dis* 21:194–200
- Rudd MR, Gellert AR, Studdy PR, Geddes DM (1983) Inhibition of exercise-induced asthma by an orally absorbed mast cell stabilizer (M&B 22, 948). *Br J Dis Chest* 77:78–86
- Russo LL, Lebel LA, Koe RB (1987) Effects of selected phosphodiesterase (PDE) inhibitors on calcium-independent PDE activity and rolipram binding sites of cerebral cortex. *Soc Neurosci Abstr* 13:903
- Salter HH (1860) Asthma. Its pathology and treatment. Churchill, London
- Sanz MJ, Cortijo J, Taha MA, Cerdá-Nicolás M, Schatton E, Burgbacher B, Klar J, Tenor H, Schudt C, Issekutz AC, Hatzelmann A, Morcillo EJ (2007) Roflumilast inhibits leukocyte-endothelial cell interactions, expression of adhesion molecules and microvascular permeability. *Br J Pharmacol* 152:481–492
- Schade FU, Schudt C (1993) The specific type III and IV phosphodiesterase inhibitor zardaverine suppresses formation of tumor necrosis factor by macrophages. *Eur J Pharmacol* 230:9–14
- Schermuly RT, Ghofrani HA, Enke B, Weissmann N, Grimminger F, Seeger W, Schudt C, Wallmarath D (1999) Low-dose systemic phosphodiesterase inhibitors amplify the pulmonary vasodilatory response to inhaled prostacyclin in experimental pulmonary hypertension. *Am J Respir Crit Care Med* 160:1500–1506

- Schmiechen R, Schneider HH, Wachtel H (1990) Close correlation between behavioural response and binding *in vivo* for inhibitors of rolipram-sensitive phosphodiesterase. *Psychopharmacology* 102:17–20
- Schneider HH, Schmiechen R, Brezinski M, Seidler J (1986) Stereospecific binding of the antidepressant rolipram to brain protein structures. *Eur J Pharmacol* 127:105–115
- Schroeder Wv (1887) Ueber die Wirkung des Coffeins als Diuretikum. *Arch Exp Path Pharmacol* 22:39–61
- Schudt C, Winder S, Eltze M, Kilian U, Beume R (1991a) Zardaverine: a cyclic AMP specific PDE III/IV inhibitor. *Agents Actions (Suppl)* 34:379–402
- Schudt C, Winder S, Fordekinz S, Hatzelmann A, Ullrich V (1991b) Influence of selective phosphodiesterase inhibitors on human neutrophil functions and levels of cAMP and Ca_i. *Naunyn-Schmiedeberg's Arch Pharmacol* 344:682–690
- Schudt C, Winder S, Mueller B, Ukena D (1991c) Zardaverine as a selective inhibitor of phosphodiesterase isozymes. *Biochem Pharmacol* 42:153–161
- Schudt C, Tenor H, Wendel A, Eltze M, Magnussen H, Rabe KF (1993) Influence of the PDEIII/IV inhibitor B9004-070 on contraction and PDE activities in airway and vascular smooth muscle. *Am Rev Respir Dis* 147:A183
- Semmler J, Wachtel H, Endres S (1993) The specific type IV phosphodiesterase inhibitor rolipram suppresses tumor necrosis factor- α production by human mononuclear cells. *Int J Immunopharmac* 15:409–413
- Seybold J, Newton R, Wright L, Finney PA, Suttrop N, Barnes PJ, Adcock IM, Giembycz MA (1998) Induction of phosphodiesterases 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and G_s-coupled receptor agonists. *J Biol Chem* 273:20575–20588
- Simet SM, Sisson JH, Pavlik JA, Devasure JM, Boyer C, Liu X, Kawasaki S, Sharp JG, Rennard SI, Wyatt TA (2009) Long-term cigarette smoke exposure in a mouse model of ciliated epithelial cell function. *Am J Respir Cell Mol Biol* 43(6):635–640
- Souness JE (1996) Characterization of different states of PDE4 by rolipram and RP 73401. In: Schudt C, Dent G, Rabe KF (eds) *Phosphodiesterase inhibitors*. Academic, London
- Souness JE, Griffin M, Maslen C, Ebsworth K, Scott LC, Pollock K, Palfreyman N, Karlsson J-A (1996) Evidence that cyclic AMP phosphodiesterase inhibitors suppress TNF generation from human monocytes by interacting with a “low affinity” phosphodiesterase 4 conformer. *Br J Pharmacol* 118:649–658
- Souness JE, Houghton C, Sardar N, Withnall MT (1997) Evidence that cyclic AMP phosphodiesterase inhibitors suppress interleukin-2 release from murine splenocytes by interacting with a “low-affinity” phosphodiesterase 4 conformer. *Br J Pharmacol* 121:743–750
- Stawiski MA, Rusin LJ, Burns TL, Weinstein GD, Voorhees JJ (1979) Ro 20-1724: an agent that significantly improves psoriatic lesions in double-blind clinical trials. *J Invest Dermatol* 73:261–263
- Takeyama K, Jung B, Shim JJ, Burgel PR, Dao-Pick T, Ueki IF, Protin U, Kroschel P, Nadel JA (2001) Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. *Am J Physiol Lung Cell Mol Physiol* 280:L165–72
- Tenor H, Schudt C (1996) Analysis of PDE isoenzyme profiles in cells and tissues by pharmacological methods. In: Schudt C, Dent G, Rabe KF (eds) *Phosphodiesterase inhibitors*. Academic, London
- Tenor H, Hatzelmann A, Wendel A, Schudt C (1995a) Identification of phosphodiesterase IV activity and its cyclic adenosine monophosphate-dependent up-regulation in a human keratinocyte cell line (HACAT). *J Invest Dermatol* 105:70–74
- Tenor H, Staniciu L, Schudt C, Hatzelmann A, Wendel A, Djukanovic R, Church MK, Shute JK (1995b) Cyclic nucleotide phosphodiesterases from purified human CD4⁺ and CD8⁺ T lymphocytes. *Clin Exp Allergy* 25:616–624
- Tenor H, Hatzelmann A, Kupferschmidt R, Staniciu L, Djukanovic R, Schudt C, Wendel A, Church MK, Shute JK (1995c) Cyclic nucleotide phosphodiesterase isoenzyme activities in human alveolar macrophages. *Clin Exp Allergy* 25:625–6333

- Tenor H, Hatzelmann A, Church MK, Schudt C, Shute JK (1996) Effects of theophylline and rolipram on leukotriene C₄ (LTC₄) synthesis and chemotaxis of human eosinophils from normal and atopic subjects. *Br J Pharmacol* 118:1727–1735
- Tenor H, Burgbacher B, Schudt C, Hatzelmann A (2005) Effects of roflumilast and other PDE4 inhibitors on human CD8+ T-cell functions. *Eur Respir J Suppl* 26:717s
- Tenor H, Bundschuh D, Schudt C, Bredenbröker D, Hatzelmann A (2007) Phosphodiesterase 4 inhibitors in the treatment of COPD. In: Stockley RS, Renard SI, Rabe KF, Celli B (eds) *Chronic obstructive pulmonary disease*. Blackwell, Oxford
- Thompson WJ, Appleman MM (1971) Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* 10:311–316
- Thompson WJ, Ross CP, Pledger WJ, Strada SJ, Banner RL, Hersh EM (1976) Cyclic adenosine 3':5'-monophosphate phosphodiesterase: distinct forms in human lymphocytes and monocytes. *J Biol Chem* 251:4922–4929
- Thompson WJ, Terasaki WL, Epstein PM, Strada SJ (1979) Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. *Adv Cyclic Nucl Res* 10:69–92
- Thorne JR, Broadley KJ (1994) Adenosine-induced bronchoconstriction in conscious hyperresponsive and sensitized guinea-pigs. *Am J Respir Crit Care Med* 149:392–399
- Thusu KJ, Morin FC, Russell JA, Steinhorn RA (1995) The cGMP phosphodiesterase inhibitor zaprinast enhances the effect of nitric oxide. *Am J Respir Crit Care Med* 152:1605–1610
- Togo S, Liu X, Wang X, Sugiura H, Kamio K, Kawasaki S, Kobayashi T, Ertl RF, Ahn Y, Holz O, Magnussen H, Fredriksson K, Skold CM, Rennard SI (2009) The PDE4 inhibitors roflumilast and rolipram augment PGE₂ inhibition of TGFβ₁ stimulated fibroblasts. *Am J Physiol Lung Cell Mol Physiol* 296:L959–L969
- Tomkinson A, Karlsson J-A, Raeburn D (1993) Comparison of the effects of selective inhibitors of phosphodiesterase type III and IV in airway smooth muscle with differing β-adrenoceptor subtypes. *Br J Pharmacol* 108:57–61
- Torphy TJ, Cieslinsky LB (1989) Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isoenzymes in canine tracheal smooth muscle. *Mol Pharmacol* 37:206–214
- Torphy TJ, Udem BJ (1991) Phosphodiesterase inhibitors: new opportunities for treatment of asthma. *Thorax* 46:512–523
- Torphy TJ, Burman M, Huang LB, Tucker SS (1988) Inhibition of the low Km cyclic AMP phosphodiesterase in intact canine trachealis by SKF 94863: mechanical and biochemical responses. *J Pharmacol Exp Ther* 246:843–850
- Torphy TJ, Zhou HL, Cieslinsky LB (1992a) Stimulation of beta adrenoceptors in a human monocyte cell line (U937) up-regulates cyclic AMP-specific phosphodiesterase activity. *J Pharmacol Exp Ther* 263:1195–1205
- Torphy TJ, Stadel JM, Burman M, Cieslinsky LB, McLaughlin MM, With JR, Livi GP (1992b) Coexpression of human cAMP-specific phosphodiesterase activity and high affinity rolipram binding in yeast. *J Biol Chem* 267:1796–1804
- Torphy TJ, Udem BJ, Cieslinsky LB, Luttmann MA, Reeves ML, Hay DW (1993) Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle. *J Pharmacol Exp Ther* 265:1213–1223
- Trendelenburg O (1912) Physiologische und pharmakologische Untersuchungen an der isolierten Bronchialmuskulatur. *Arch Exp Path Pharmacol* 69:79–107
- Tudor RM, Voelkel NF (2002) The pathobiology of chronic bronchitis and emphysema. In: Voelkel NF, MacNee W (eds) *Chronic obstructive lung disease*. BC Decker, London
- Underwood DC, Osborn RR, Novak LB, Matthews JK, Newsholme SJ, Udem BJ, Hand JM, Torphy TJ (1993) Inhibition of antigen-induced bronchoconstriction and eosinophil infiltration in the guinea pig by the cAMP-specific phosphodiesterase inhibitor rolipram. *J Pharmacol Exp Ther* 266:306–313
- Underwood DC, Kotzer CJ, Bochnovicz S, Osborn RR, Luttmann MA, Hay DWP, Torphy TJ (1994) Comparison of Phosphodiesterase III, IV and dual III/IV inhibitors on

- bronchospasm and pulmonary eosinophil influx in guinea pigs. *J Pharmacol Exp Ther* 270:250–259
- Vergheze MW, McConnell RT, Lenhard JM, Hamacher L, Jin C (1995) Regulation of distinct cyclic AMP-specific phosphodiesterase (phosphodiesterase type 4) isozymes in human monocytic cells. *Mol Pharmacol* 47:1164–1171
- Virag R, Zwang G, Dermange H, Legman M (1981) Vasculogenic impotence: a review of 92 cases in 54 surgical operations. *Vasc Surg* 15:9–17
- Vogel HG (2002) Drug discovery and evaluation. Springer, Heidelberg
- Wachtel H (1983) Species differences in behavioural effects of rolipram and other adenosine cyclic 3, 5-monophosphate phosphodiesterase inhibitors. *J Neural Transm* 56:139–152
- Wachtel H, Schneider HH (1986) Rolipram, a novel antidepressant drug, reverses the hypothermia and hypokinesia of monoamine-depleted mice by an action beyond postsynaptic monoamine receptors. *Neuropharmacology* 25:1119–1126
- Weidenbach A, Braun C, Schwoebel F, Beume R, Marx D (2008a) Steroid-insensitivity in a short-term model of cigarette smoke-induced pulmonary inflammation in mice. *Am J Respir Crit Care Med (Suppl)* A651 (abstract)
- Weidenbach A, Braun C, Schwoebel F, Beume R, Marx D (2008b) Therapeutic effects of various PDE4 inhibitors on cigarette smoke-induced pulmonary neutrophilia in mice. *Am J Respir Crit Care Med (Suppl)* A651 (abstract)
- Weishaar RE, Cain MH, Bristol JA (1985) A new generation of phosphodiesterase inhibitors: multiple molecular forms of phosphodiesterase and the potential for drug selectivity. *J Med Chem* 28:537–545
- Weisshaar RE, Quade M, Boyd D, Schenden J, Marks S, Kaplan HR (1983) The effect of several “new and novel” cardiotonic agents on key subcellular processes involved in the regulation of myocardial contractility: implications for mechanism of action. *Drug Dev Res* 3:517–534
- Wetzel B, Huel N (1988) New cardiotonic agents – a promising approach for treatment of heart failure. *Trends Pharmacol Res* 9:166–170
- Wilkens JH, Neuenkirchen H, Sybrecht GW, Oellerich M (1984) Individualizing theophylline dosage: evaluation of a single-point maintenance dose prediction method. *Eur J Clin Pharmacol* 26:491–498
- Wohlsen A, Wollin L, Marx D, Beume R (2006). Effect of roflumilast and other cAMP elevating agents on airway beat ciliary frequency in proximal and distal airways in rat precision cut lung slices. Fifth international multidisciplinary Conference on Chronic Obstructive Pulmonary Disease (COPD5), June 28–30, Birmingham, UK: Poster P25.

Phosphodiesterase Inhibitors: Factors That Influence Potency, Selectivity, and Action

Sharron H. Francis, Miles D. Houslay, and Marco Conti

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Abstract Cyclic nucleotide phosphodiesterases (PDEs) are promising targets for pharmacological intervention. The presence of multiple PDE genes, diversity of the isoforms produced from each gene, selective tissue and cellular expression of the isoforms, compartmentation within cells, and an array of conformations of PDE proteins are some of the properties that challenge the development of drugs that target these enzymes. Nevertheless, many of the characteristics of PDEs are also viewed as unique opportunities to increase specificity and selectivity when designing novel compounds for certain therapeutic indications. This chapter provides a summary of the major concepts related to the design and use of PDE inhibitors. The overall structure and properties of the catalytic domain and conformations of PDEs are summarized in light of the most recent X-ray crystal structures. The distinctive properties of catalytic domains of different families as well as the technical challenges associated with probing PDE properties and their interactions with small molecules are discussed. The effect of posttranslational modifications and protein–protein interactions are additional factors to be considered when designing PDE inhibitors. PDE inhibitor interaction with other proteins needs to be taken into account and is also discussed.

Keywords Compartmentation · Cyclic AMP · Cyclic GMP · PDE1 · PDE2 · PDE3 · PDE4 · PDE5 · PDE6 · PDE7 · PDE8 · PDE9 · PDE10 · PDE11 · Phosphodiesterase · Phosphodiesterase inhibitors

1 PDE Superfamily

The mammalian superfamily of cyclic nucleotide (cN) phosphodiesterases (PDEs) is remarkably complex. It comprises 11 distinct families (PDEs 1–11) with protein products derived from 21 genes (Bender and Beavo 2006; Conti and Beavo 2007); some families are encoded by a single gene, whereas others are products of multiple genes, but there are alternative splice variants of the gene products in all the families except for PDE6. In several instances, multiple promoters that are differentially regulated influence expression of the PDE mRNA transcripts (Bender and Beavo 2006; Conti and Beavo 2007; Omori and Kotera 2007), and extensive alternative splicing of the mRNAs produces a vast array of protein products. It is now estimated that there are close to 100 different protein products of these genes, and these are distinguished by having different regulatory features, catalytic characteristics, tissue distributions, subcellular localizations, targeting to signaling complexes and sensitivities to PDE inhibitors (Fig. 1). The need for such a large array of PDE isoforms in controlling cN levels and maintaining appropriate cellular functions is still poorly understood. PDEs are typically in low abundance in cells but may be highly expressed in particular tissues or regions of cells for regulation of specific physiological effects (Castro et al. 2006; Cote 2006; Houslay 2010; Juilfs et al. 1997; MacFarland et al. 1991). Most cells contain multiple PDEs that have overlapping specificities and affinities for cAMP or cGMP. However, where studied, it seems clear that while there may be some degree of redundancy in function, each of the PDEs provides important regulatory control of cNs in a particular cell or region of

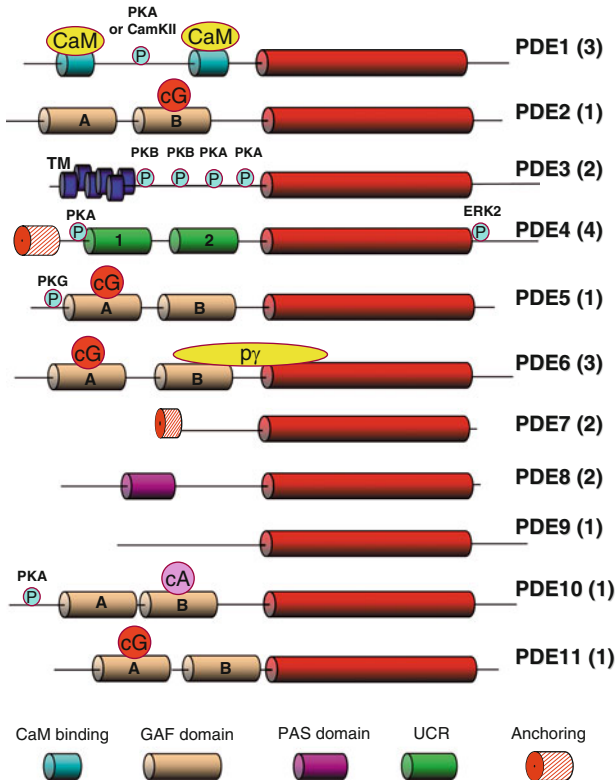


Fig. 1 Schematic representation of domain arrangement of the 11 mammalian PDE families. Family name is noted to the *right* of each structure, and the *number in parenthesis* denotes the number of genes composing the family. The conserved catalytic domain is represented as a *red cylinder*. Binding proteins are depicted in *yellow*. TM = transmembrane domain of PDE3; protein kinase (PKB, PKA, PKG, ERK, or CaMKII) phosphorylation sites are shown as a *teal ball* labeled with a P. Cyclic GMP binding to either GAF-A or GAF-B domains is marked by a *red ball* labeled cG, and cAMP binding to GAF-B in PDE10 is depicted as a *pink ball* labeled cA. Modified from Conti and Beavo (2007)

a cell. Some PDE families (PDEs 1–4) are widely expressed in mammalian tissues, but others (PDEs 5–11) occur in lower abundance or are expressed in fewer tissues. The PDE6 family appears to be the most restricted in distribution since it has only been found in the outer segments of photoreceptors, where it is in high concentration, and in the pineal gland (Cote 2006).

1.1 PDEs as Cellular Targets of Cyclic Nucleotides

The myriad forms of PDEs that serve as cellular targets of cNs and as major determinants of cN action exceed those of other targets such as that of cN-dependent

protein kinases, the cN-gated (CNG) channels and exchange-protein activated by cAMP (EPAC). All PDEs contain a conserved catalytic site that interacts with cNs and breaks them down into their respective 5'-nucleotides. Several PDEs (PDE 2, 5, 6, 10, and 11) contain cN-binding sites in their respective regulatory domains (Cote 2006; Gross-Langenhoff et al. 2006; Wu et al. 2004; Zoraghi et al. 2005; Handa et al. 2008; Martinez 2002a; Martinez 2002b; Martinez et al. 2008); these sites comprise ~120 amino acids known as GAFs [an acronym derived from the proteins in which these domains were originally identified, i.e., cGMP-binding PDEs, *Anabaena* adenylyl cyclases, and *Escherichia coli* transcription factor FhlA (Aravind and Ponting 1997)]. For PDE 2, 5, and 6, cN binding to one of these GAFs regulates catalytic site function, and it has been proposed that cN-binding GAFs in PDEs could act to sequester cNs under appropriate conditions (Bender and Beavo 2006; Conti and Beavo 2007; Corbin and Francis 1999; Gopal et al. 2001).

The respective cN-binding sites in GAFs are structurally and evolutionarily distinct from the PDE catalytic sites and cN-binding sites in the cN-dependent protein kinases, cN-regulated channels, the bacterial catabolite-gene activator protein (CAP), and EPACs (Bos 2006; Charbonneau 1990; Martinez et al. 2002a, b; Zoraghi et al. 2004). The regulatory (R) and catalytic (C) subunits of cAMP-dependent protein kinase (PKA), which combine to form the PKA holoenzymes, are derived from four and three genes, respectively. Any R subunit (RI or RII), which appear to exist as homodimers, can interact with any C subunit (C α , C β , or C γ) in mammalian tissues (Francis and Corbin 1999). EPACI and II, which are regulated by cAMP binding, are derived from two genes (Bos 2006), and cGMP-dependent protein kinases (PKGI and PKGII) are derived from two genes with alternative splicing of the PKGI mRNA to produce two isoenzymes (PKGI α and PKGI β) (Uhler 1993; Wernet et al. 1989). PKGs appear to always exist as homodimers so it is predicted that there are only three PKGs in mammalian tissues. The exceptionally diverse characteristics of the PDEs provide excellent potential for development of selective inhibitors for these targets, but in most instances, their similarities continue to confound development of such inhibitors.

The allosteric cN-binding sites on PDEs 2, 5, 6, and 11 preferentially bind cGMP, although the sites on PDE2 can interact with cAMP with reasonable affinity (Martinez et al. 2002a, b; Wu et al. 2004; Zoraghi et al. 2005; Cote 2006); the allosteric cN-binding site in PDE10 tightly binds cAMP (Handa et al. 2008). When compared with other cN-binding sites, these sites are formed by a tight binding pocket; in PDEs 5 and 6, this pocket rigorously selects for cGMP versus cAMP and against substituents introduced in cGMP analogs (Huang et al. 2004; Martinez 2002; Thomas et al. 1992; Wu et al. 2004). These characteristics make them excellent targets for drugs that would impact the function of these PDEs. In PDEs 5 and 6, the allosteric site excludes PDE inhibitors. However, in PDE2, low concentrations of 3-isobutyl-1-methylxanthine (IBMX) or papaverine stimulate catalytic activity (Yamamoto et al. 1983), although it is now unclear whether this stimulation occurs through interaction with the allosteric cN-binding site or effects mediated via partial occupation of the catalytic site (Pandit et al. 2009). If this effect is mediated through the allosteric cN-binding site, it lends some promise for

development of compounds that selectively interact with PDE2 allosteric sites (Erneux et al. 1982; Yamamoto et al. 1983). Recent insights derived from X-ray crystallographic and NMR structures of several cN-binding GAFs have further defined topographical features that could lead to design of pharmacophores targeting these sites (Heikaus et al. 2008; Martinez et al. 2005; Pandit et al. 2009; Wang et al. 2011), and high throughput screening assays to identify compounds that might interact with these sites are currently being developed (see Demirbas et al. and Schultz et al. in Chapters 5 and 6, respectively, in this volume). Likewise, the *Upstream Conserved Regions* (UCRs), which profoundly impact catalytic functions in PDE4 isoforms, and the Rec and PAS subdomains in PDE8 are considered to be promising pharmacological targets for modulation of the catalytic activities of these PDEs.

1.2 Quaternary Structure of PDEs

PDEs exist as monomers, dimers, or higher oligomers, and in most instances, the contribution of this physical status to function, regulation, localization, and stability is poorly understood. Most PDEs appear to exist as homodimers except in the case of PDEs 1 and 6. Under physiological conditions, PDE1 is thought to occur as a heterotetramer comprising two identical catalytic subunits and two molecules of calmodulin. PDE6 isoforms in the outer segments of the photoreceptors also commonly exist as heterotetramers. The PDE6 in rod outer segments is composed of two closely related gene products (PDE6 α and PDE6 β) that form a catalytic heterodimer that is in complex with two small rod-specific inhibitory subunits (P γ) to form an inactive heterotetramer (Cote 2006). The PDE6 in cone outer segments is also a heterotetramer that comprises two identical catalytic subunits (PDE6 α') and two cone-specific P γ subunits.

The mechanisms employed for oligomerization of PDEs vary; some interact through their catalytic domains (Huai et al. 2003; Scapin et al. 2004), others make contacts through portions of their regulatory domains including GAFs or UCRs (Huai et al. 2003; Martinez 2002; Richter and Conti 2002; Zoraghi et al. 2005), and still others dimerize through contacts in both regions (Pandit et al. 2009). In all instances, the contacts and quaternary state appear to be very stable. For most PDEs, catalytic activity is retained in constructs that include only the catalytic domain; in several instances, these truncated constructs are monomeric indicating that oligomeric status is not required for catalytic function. For the isolated catalytic domains of some PDEs, many functional characteristics (k_{cat} , K_{m} , and IC_{50} values for certain inhibitors) are similar to those of the respective holoenzymes, but in others, there are important functional differences (Blount et al. 2006, 2007; Fink et al. 1999; Richter and Conti 2004; Saldou et al. 1998).

Almost all inhibitors that have been developed to date compete with cN substrate for access to the PDE catalytic sites. This region continues to be a major focus of medicinal chemists for development of inhibitors (Ke and Wang 2007b; Owen et al. 2009; Verhoest et al. 2009; Zhang 2006). The catalytic domain, which is conserved

among mammalian PDEs and comprises ~270 amino acids (Bender and Beavo 2006; Conti and Beavo 2007), is located toward the carboxyl-terminal portion of the PDEs and exhibits 24–51% amino acid sequence identity among the 11 families. X-ray crystal structures of the isolated catalytic domains have shown that the various PDEs share a similar overall topography that is comprised almost entirely of α -helices (16–17 helices), as well as sharing common structural features in the composition and conformation of the catalytic pockets (Ke and Wang 2007b; Zhang 2006). However, there are important differences among these proteins in the binding of cNs and inhibitors (Wang et al. 2005; Wang, Liu, Hou et al. 2007; Wang, Yan, Yang, Cai et al. 2008; Huai, Wang, Zhang et al. PNAS 2004; Huai, Liu, Francis, Corbin JBC 2004). Moreover, potent and selective inhibitors of PDE4 have recently been developed by dually targeting the catalytic sites of these enzymes together with regions outside the catalytic pocket itself (Burgin et al. 2010; Gurney et al. 2011).

1.3 Structural Characteristics

Divalent metal ions are required for catalytic function of all PDEs. In the X-ray crystallographic structures of PDEs, the catalytic pockets have been shown to contain a novel binuclear metal-binding site that is thought to contribute critical elements of the catalytic mechanism (Xu et al. 2000). Where it has been studied, the interactions of certain inhibitors with PDE catalytic sites can be influenced by the presence of these metal ions, but direct or indirect interaction with the metals does not appear to substantially impact potency (Chen et al. 2008; Corbin et al. 2003; Ke and Wang 2007b; Sung et al. 2003; Wang et al. 2008). However, it is reasonable that inhibitors could be designed that utilize contacts with one or more of the metal ions or that expel these cofactors, thereby eliminating catalytic activity. Since affinity of certain PDE4 long isoforms for various selective inhibitors and magnesium is sensitive to PKA phosphorylation of the regulatory UCR1 domain (Alvarez et al. 1995; Sette and Conti 1996b; Hoffmann et al. 1998), it seems likely that the metal ions bound to a particular PDE could also affect its interaction with certain inhibitors. Moreover, some inhibitors could influence metal occupancy of PDE catalytic sites (Wang et al. 2008), but this possibility has not yet been investigated.

Results of X-ray crystallographic studies of certain PDEs reveal that a hydroxyl derived from solvent water bridges the two metal ions in these catalytic sites. This arrangement provides for production of a highly nucleophilic hydroxyl that inserts at the phosphate in the cyclic phosphate ring of the cN and breaks the ring (Fig. 2) (Liu et al. 2008). These data are consistent with the results of early biochemical studies establishing that the hydroxyl inserted into the cyclic phosphate ring is derived from solvent water and that the stereochemistry of the product is consistent with this mechanism (Braumann et al. 1986; Burgers and Eckstein 1979; Jarvest et al. 1982; Walseth et al. 1983). In most instances, one of the metal sites is occupied by a zinc that is very tightly bound, and the other site is occupied by a more loosely

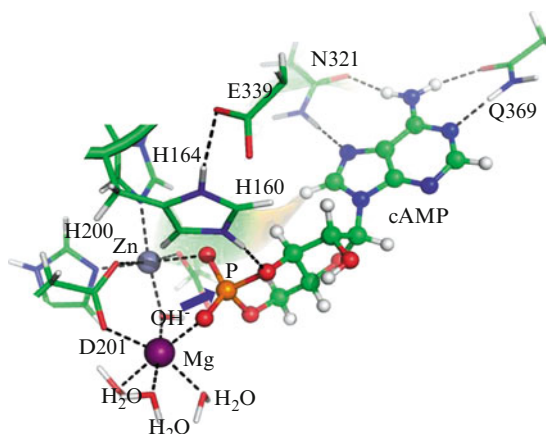


Fig. 2 Scheme proposed for mechanism of hydrolysis of cAMP by PDE4. The adenine (shown in green and blue at upper right) is coordinated through hydrogen bonds (dashed lines) to Gln369 and Asn321. The phosphorous in the cyclic phosphate ring is approximated to the hydroxyl ion that bridges the two metal ions [Zn (in steel) and Mg (in purple)], and the blue arrow indicates the attack of this hydroxyl at that phosphorous (shown in orange) to break the ring. Residues coordinating the metal ions are shown. The Glu339/His160 hydrogen-bond relay that fosters interaction of His160 with one of the oxygens (red) in the cyclic phosphate ring is shown. This figure was kindly provided by Prof. Chang-Guo Zhan, College of Pharmacy at the University of Kentucky

bound metal that is presumed to be either magnesium or manganese (Ke and Wang 2007b; Sung et al. 2003; Xu et al. 2000). The exact complement of metal ions that occupy this binuclear site has not been defined for any mammalian PDE since catalysis in various PDEs is preferentially supported by different metals including magnesium, manganese, cobalt, or zinc.

A zinc atom is clearly defined in most of the X-ray crystal structures of PDE catalytic domains and is present even when crystals are formed in the presence of chelators (Ke and Wang 2007b; Xu et al. 2000), but the metal ion occupying the second site has not been identified for any PDE. The X-ray structure of the refolded PDE3 catalytic domain is the only structure of a wild-type PDE protein determined thus far that lacks a zinc ion (Scapin et al. 2004). By direct chemical analysis, PDE6 has been shown to contain 3–4 zincs per dimer. Zinc is also critical for PDE6 catalytic function (He et al. 2000), but magnesium also stimulates PDE6 activity. Likewise, PDE5 binds ~6 zincs per dimer, and zinc at submicromolar levels support catalytic function, but manganese, cobalt, and magnesium at higher concentrations also support catalysis (Francis et al. 1994). The precise role of the respective metal ions in the catalytic function of each PDE is still not well understood. To exploit the effect of an inhibitor to interfere with the role of these metal ions in PDE functions, it will be critical to better define the particular metal complement that is important for catalytic function of a PDE isoform.

The volume of the PDE catalytic sites has been estimated to be ~330–450 Å³, and several structures of isolated catalytic domains in complex with either the

5'-nucleotide product or the selective inhibitors are now available (Ke and Wang 2007b; Wang et al. 2007; Xu et al. 2000, 2004). Interestingly, the orientation of substrate, inhibitor, or product can vary substantially among PDEs (Wang, Liu, Hou 2007). This may be due to the relatively large volume of the catalytic site compared to the size of the substrate/product or most inhibitors. However, this variation restricts the potential for generalizations from one structure to another. Recently, X-ray crystal structures of two PDEs containing either the near-full length regulatory domain (PDE2) (Pandit et al. 2009) or a portion thereof (PDE4) (Burgin et al. 2010) have been published. Both structures provide important new insights into regulatory domain functions, and the report on the PDE4 constructs reveals novel new approaches to inhibitor design and action (see below and in Gurney et al. in Chapter 7 in this volume). However, the X-ray crystal structure of PDE2 defines the structure in the absence of cGMP, that is, the lower activity state of the enzyme, and provides only a partial picture of the functional states of PDE2 (Pandit et al. 2009). In X-ray crystal structures of isolated catalytic domains in complex with various inhibitors, substrates, or catalytic products, hydrogen bonding with an invariant glutamine and hydrophobic stacking of the ring structure of the substrate/product/inhibitor with a conserved phenylalanine (in most PDEs) are common interactions (Fig. 2) (Ke and Wang 2007a, b; Xu et al. 2000, 2004). Another group of amino acids in the catalytic pocket forms a hydrophobic face that wedges the ring structure of the ligands against the conserved phenylalanine, thereby creating what has been termed a "hydrophobic clamp." These interactions occur for most PDEs when associated with a wide spectrum of inhibitors/products that vary significantly in affinities and chemical characteristics (Ke and Wang 2007b).

For several PDE holoenzymes, the energy contribution of amino acids in the catalytic pockets to the affinity for substrate or inhibitors have been quantified using site-directed mutagenesis (Burgin et al. 2010; Cheung et al. 1998; Jacobitz et al. 1996; Jin et al. 1992; Omburo et al. 1998; Turko et al. 1999; Wang et al. 2005; Zhang et al. 2002; Zoraghi et al. 2007). It is evident from the X-ray crystallography and mutagenesis studies that different inhibitors exploit novel features in and near the catalytic sites of the respective PDEs to enhance potency and selectivity (Ke and Wang 2007b; Sung et al. 2003; Wu et al. 2004). In some instances, enhanced specificity/potency is provided by sequence(s) well outside the catalytic domain (Blount et al. 2006; Burgin et al. 2010; McPhee et al. 1999; Omori and Kotera 2006; Richter and Conti 2004; Saldou et al. 1998), but currently insights into the mechanisms that provide for this are limited.

1.4 Functional Distinctions Among PDE Catalytic Sites

1.4.1 Catalytic Characteristics

Despite strong structural similarities among the PDE catalytic sites, PDEs 4, 7, and 8 are highly specific for hydrolysis of cAMP, PDEs 5, 6, and 9 are highly specific

for hydrolysis of cGMP, and others (PDEs 1, 2, 3, 10, and 11) hydrolyze both cNs. Moreover, functional features of closely related PDEs can differ substantially, a result that is encouraging for development of specific inhibitors for each family. Some PDEs that exhibit the highest identity in amino acid sequence, for example, PDEs 5 and 11 (catalytic domains have ~51% sequence identity) have very different selectivities for cAMP and cGMP (Bender and Beavo 2006; Omori and Kotera 2006, 2007). PDE5 exhibits ~100-fold greater affinity for cGMP than for cAMP, although both are hydrolyzed at ~equal rates (Francis and Corbin 2009). In contrast, PDE11 hydrolyzes both cNs with similar affinities and efficiencies. In addition, PDEs 5 and 11 exhibit very different affinities for potent PDE5 inhibitors currently in clinical use, that is, vardenafil, sildenafil, and tadalafil; potencies of these compounds for PDE5 compared to PDE11 differ by 7,000-, 950-, and 41-fold, respectively (Weeks et al. 2007).

In another example, the amino acid sequence identity of the catalytic domains of PDEs 5 and 6, both of which are highly specific for cGMP, are very similar (~42%), but the affinity of PDE6 catalytic site for cGMP ($K_m \sim 14 \mu\text{M}$), is seven times weaker than that of PDE5 (~2 μM), and the catalytic rate of PDE6 (~2,000 $\mu\text{mol}/\text{min}/\text{mg}$) exceeds that of PDE5 by ~1,000-fold (Cote 2006; Francis et al. 2006). Moreover, PDE6, like PDE5, is potently inhibited by sildenafil, vardenafil, and zaprinast, but tadalafil, a potent PDE5 inhibitor, is a weak inhibitor of PDE6 (Zhang 2006). These data imply that subtle differences in the topography and chemical characteristics of the active site can have profound effects on substrate preference, catalytic efficiency, and inhibitor potencies.

In some instances, there are even significant distinctions among catalytic sites of PDE isoforms within the same family. For example, PDE1A, PDE1B, and PDE1C are products of three separate genes with catalytic domains that share ~75% sequence homology but have quite different selectivity for cGMP ($K_m = 1 \mu\text{M}$ for PDE1C2, 3 μM for PDE1B1, and 5 μM for PDE1A2), compared to that for cAMP ($K_m = 1 \mu\text{M}$, 24 μM , and 113 μM , respectively). Nevertheless, the maximum catalytic activities of these PDEs for breakdown of cGMP and cAMP are similar (Bender and Beavo 2006).

1.4.2 Potencies of Inhibitors Within PDE Families

The potency of an inhibitor for PDEs within a family, among splice variants within a subfamily, or between cytosolic and membrane-bound forms of the same PDE can also differ significantly. For example, vinpocetine, a selective PDE1 inhibitor, more potently blocks the catalytic activity of PDE1A and PDE1B than that of PDE1C (Yan et al. 1996). The inhibitory potency of IC86340, another selective PDE1 inhibitor, varies by ~7-fold among PDE1 isoforms; IC_{50} values are: PDE1C (0.06 μM), PDE1B (0.21 μM), PDE1A (0.44 μM) (Miller et al. 2009). Moreover, potency of another PDE1-selective inhibitor, SCH51866, for several alternative

splice variants of PDE1C differs by threefold: PDE1C1 ($IC_{50} = 101$ nM) versus PDE1C4/5 ($IC_{50} = 36$ nM) (Yan et al. 1996).

PDE4 inhibitors can also exhibit quite different potencies for the various members of this family. Cilomilast, a highly selective inhibitor of the PDE4 family, exhibits significantly higher potency (7- to 27-fold) toward PDE4D ($IC_{50} \sim 12$ nM) compared to that for PDE4A ($IC_{50} \sim 115$ nM), PDE4B ($IC_{50} \sim 86$ nM), or PDE4C ($IC_{50} \sim 308$ nM) (Torphy 1998). Inhibitors that show good selectivity for PDE4D over PDE4B have also been identified: NVP-ABE171 is 20-fold selective for PDE4D over PDE4B (Trifilieff et al. 2002) and CP-671305 is 95-fold selective for PDE4D over PDE4B (Kalgutkar et al. 2004; Zhang 2006). Moreover, D159687, a compound recently reported by investigators at deCode Genetics, is highly selective for PDE4D7 versus PDE4A1, PDE4B1, and PDE4C1 (93-, 54-, and 250-fold difference in potency, respectively) (Burgin et al. 2010). The structural subtleties that provide for these differences in such closely related enzymes are typically not fully understood; they could relate to differences in the catalytic site pocket or reflect the influence of interactions of the inhibitors with regions outside the catalytic pocket as occurs for the D159687 compound or interaction with protein binding partners and posttranslational modification (Burgin et al. 2010; Houslay 2001; Houslay and Adams 2003; Houslay et al. 2005, 2007). Exploiting these differences may open novel opportunities for the development of more selective inhibitors. However, each modest advance in devising a compound that shows selectivity among PDEs is just the beginning in the efforts to develop a compound with pharmacokinetic and biochemical features suitable for clinical use.

2 Factors That Impact Catalytic Site Function and Potency of Inhibitor Action

PDE catalytic site functions can be modified by many processes, including post-translational modifications, change in the cellular milieu (pH or redox conditions), interaction with activators (e.g., calcium/calmodulin, cGMP, or phosphatidic acid), binding to other proteins, and exposure to drugs (Burgin et al. 2010; Houslay 2001; Houslay and Adams 2003, 2010; Houslay et al. 2005, 2007). The influence of these factors on the potencies of inhibitors for any PDE is not fully understood and effects identified for a particular inhibitor frequently do not apply to other inhibitors of that PDE family. Consequently, environmental or posttranslational effects on each inhibitor must be assessed independently. The state of oligomerization of a PDE may also be a determinant of the pharmacological properties of an inhibitor (Burgin et al. 2010; Richter and Conti 2004; Rybalkina et al. 2010). Moreover, it is clear that insights derived from structural studies of isolated catalytic domains of PDEs do not always reveal all of the important contact points that could be exploited in the design of new inhibitors (Blount et al. 2006; Burgin et al. 2010).

2.1 *Comparison of Native and Recombinant Enzymes*

A recurrent finding when investigating the properties of various PDEs is that the kinetic behavior of a recombinant, ectopically expressed enzyme does not entirely overlap with that of its endogenous native counterpart (Bolger et al. 1993; Livi et al. 1990; McPhee et al. 1999; Obernolte et al. 1993; Salanova et al. 1998; Saldou et al. 1998; Sullivan et al. 1994; Wang et al. 1997). The differences include affinity for substrate, catalytic efficiency, and inhibitor potencies (Obernolte et al. 1993; Salanova et al. 1998; Wang et al. 1997). Certainly, some differences are due to the fact that native PDE preparations are often heterogeneous in terms of enzyme composition (Obernolte et al. 1993). For many PDEs, the physicochemical properties of the various isoforms are not sufficiently different to allow for complete separation with even the most current chromatographic methods. More frequently, differences are associated with the heterologous expression system used (i.e., bacteria, yeast, insect cells, or mammalian cell lines) and the posttranslational modification of the recombinant proteins (Hoffmann et al. 1998; Saldou et al. 1998). Aberrant phosphorylations, different states of phosphorylation, or other modifications such as ubiquitination or proteolytic cleavage of overexpressed proteins have all been reported for heterologously expressed PDEs. Heterogeneity in the conformation of some recombinant proteins is an additional major variable. Overexpression of recombinant PDEs often produces proteins that are improperly folded, aggregated, or catalytically inactive (Rocque et al. 1997; Scapin et al. 2004; Wang et al. 1997). The presence of catalytic domains in different conformations due to posttranslational modification and interaction with partner proteins can confound the kinetics of inhibition of enzyme preparations with potencies reported for rolipram inhibition of PDE4 isoforms ranging from 0.01 to 10 μM (Hoffmann et al. 1998; Houslay and Adams 2003; Huston et al. 1996; Laliberte et al. 2000; McPhee et al. 1999; Rocque et al. 1997; Salanova et al. 1998; Tian et al. 1998). Proteolysis of overexpressed enzymes may also alter interaction with inhibitors without affecting catalytic activity (see below).

2.2 *Truncated Versus Full-Length PDEs*

An additional property to consider is whether truncated forms of PDEs recapitulate the properties of the full-length enzymes. Two examples involving PDEs 4 and 5 are provided to expand upon this concept. Rolipram, as well as some other compounds, inhibits isolated catalytic domains of PDE4s with potencies that are often 10- to 100-fold lower than those obtained with full-length enzyme (Jacobitz et al. 1996; Richter and Conti 2004; Tian et al. 1998). Progressive truncation at the amino terminus of PDE4B and PDE4A causes a parallel decrease in the affinity for rolipram (Jacobitz et al. 1996; Richter and Conti 2004; Rocque et al. 1997; Saldou et al. 1998). The potencies of three inhibitors (RS-25344, rolipram, and TVX 2706) for inhibition of the isolated catalytic domain of PDE4D are much weaker than for

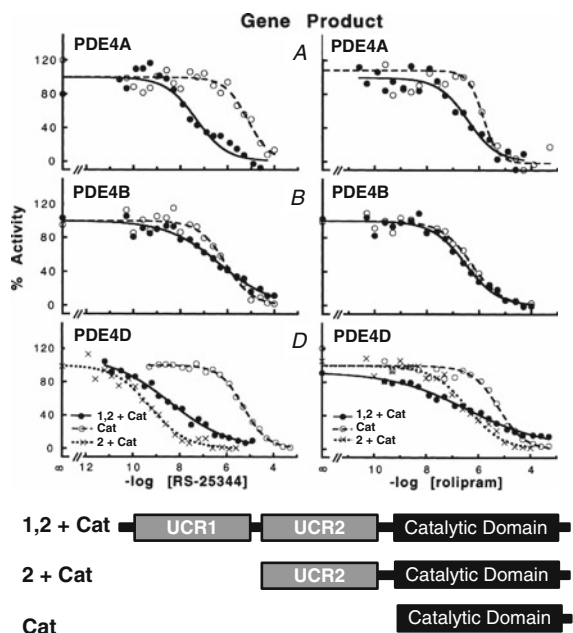


Fig. 3 Comparison of the potency of inhibition of cloned forms of PDE4 gene products with rolipram and RS-25344. Representative data showing the inhibition of PDE4A, 4B or 4D activity (*gene product A, B and D*, respectively) by RS-25344 (*left*) or rolipram (*right*). A cartoon model depicts the functional domains in the respective long (1,2 + Cat) forms of PDE4 proteins containing UCR1, UCR2, and the catalytic domain, the short (2 + Cat) forms of the enzymes containing UCR2 and the catalytic domain, as well as that of the isolated catalytic domain (Cat). Each data point represents the average of quadruplicates, and the curves are plotted as a percentage of the difference between the maximum and minimum rates of each enzyme. Reprinted from Saldou et al. (1998)

the full-length enzyme, but stronger potencies are exhibited in constructs where UCR2 is conjoined with the catalytic domain (Fig. 3) (Saldou et al. 1998). Likewise, Burgin and coworkers recently reported that two inhibitors (RS25344 and PMNPQ) are 10,000 times more potent toward inhibition of PDE4D7 than against the isolated PDE4 catalytic domain (Burgin et al. 2010). It is now clear that these truncations remove important regulatory/oligomerization domains in the holoenzyme that affect interdomain contacts and impact the conformation and/or functions of the catalytic domain (Burgin et al. 2010; Houslay 2001; Houslay and Adams 2003, 2010; Richter and Conti 2002). It has been observed that some variants of PDE4s, termed short forms, have lower affinity for rolipram and kinetic properties distinct from those of longer forms (see below). Since short and long forms are expressed in a tissue- and cell-specific manner, this must be considered when evaluating the potency and biological action of selected compounds.

Likewise, the inhibitory potency of the PDE5 inhibitor, vardenafil, for the PDE5 holoenzyme exceeds that of sildenafil by 10- to 40-fold (Blount et al. 2004, 2006).

The potency of sildenafil for inhibition of the isolated catalytic domain is essentially the same as that for the PDE5 holoenzyme, but the potency of vardenafil inhibition of the isolated catalytic domain is 10- to 40-times less than that for the holoenzyme. In fact, it has the same potency for inhibition of the isolated catalytic domain as that of sildenafil. In the X-ray crystal structures of the PDE5 catalytic domain in complex with the respective inhibitors, the contacts between each of the inhibitors and the catalytic site are very similar (Sung et al. 2003; Wang et al. 2006). The higher potency of vardenafil is retained in constructs of the catalytic domain conjoined with GAF-B indicating that in some instances structural features outside the catalytic domain contribute importantly to inhibitor potency and selectivity (Blount et al. 2006). Thus, comparing potencies of sildenafil and vardenafil, two closely related compounds, or predicting potencies using only the isolated catalytic domain would have missed important elements that contribute to the higher potency of vardenafil for the holoenzyme.

Given that many compound screens have been performed using truncated PDEs that lack their regulatory domains and because different PDE isoforms are characterized by subtle differences in conformation, this approach may have hindered identification of more selective compounds for certain PDE families and variants within PDE families.

2.3 Posttranslational Modification and Occupancy of Allosteric Sites

Posttranslational modification of a PDE may have profound effects on the potency of selected groups of compounds. This has been clearly demonstrated for both the PDE4 and the PDE5 families. PKA phosphorylation of PDE4 affects the potency of several compounds in a complex manner (Alvarez et al. 1995; Burgin et al. 2010; Hoffmann et al. 1998; Houslay and Adams 2003, 2010; Laliberte et al. 2002). PKA phosphorylation of long PDE4 isoforms, in general, increases the affinity for the prototypical inhibitor rolipram and alters the kinetics of this inhibition (Fig. 4). In the same vein, phosphorylation of PDE4D3 increases its sensitivity to inhibition by RS-25344 (~100-fold) and RS-33793 (~330-fold) and phosphorylation of PDE4A4 increases the potencies of (*R*)- and (*S*)-rolipram but does not affect the potencies of CDP-840 or SB-207499. Screening for compound potency with a phosphorylated PDE4 isoform is a strategy that has been adopted to identify compounds with high potency, but this has had little success in improving the therapeutic window of this class of compounds. Phosphorylation also modulates PDE5 enzyme functions, with detectable changes in the conformation of the enzyme (Bessay et al. 2008; Corbin et al. 2000). These changes in conformation, in turn, are associated with an increase in the affinity of PDE5 for substrate as well as for inhibitors, such as sildenafil. The concept has been proposed that when PDE5 inhibitor is present in cells, cGMP level and phosphorylation of PDE5 by PKG is increased, which in turn increases PDE5 inhibitor binding at the catalytic site, that

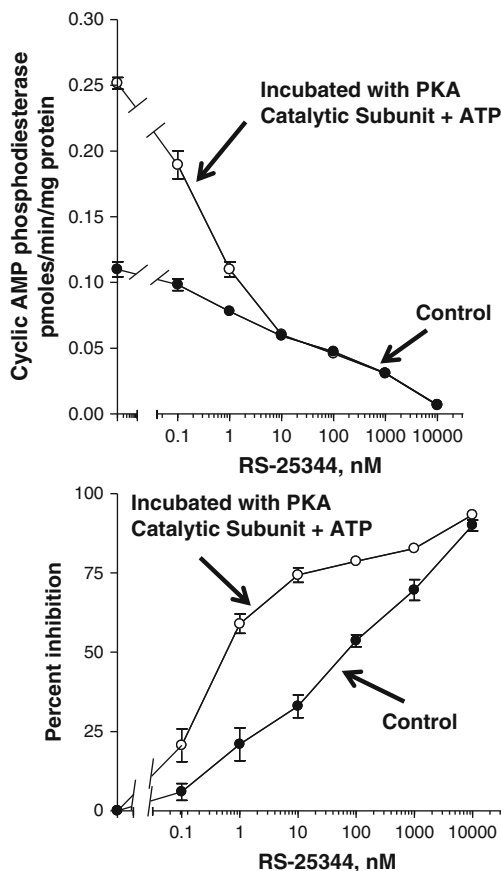


Fig. 4 Effect of phosphorylation on PDE4D3 sensitivity to inhibition by RS-25344. Phosphorylation causes a marked shift of the sensitivity of PDE4D3 to inhibition by RS25344. PDE4D3 was preincubated in the presence (*open circles*) or absence (*closed circles*) of the catalytic subunit of protein kinase and Mg/ATP. In *panel A*, the ordinate represents specific activity. In *panel B*, the ordinate represents percent inhibition calculated from the results shown in *panel A*. Values represent the value \pm standard deviation of triplicate determinations. Data presented are from a representative experiment that was repeated three times with similar results. Reproduced from Alvarez et al. (1995)

is, a feed-forward effect on inhibitor binding and potency (Bessay et al. 2008; Corbin and Francis 1999; Francis and Corbin 2005; Francis et al. 2008). A similar regulatory loop likely applies to PDE4 isoforms that are phosphorylated by PKA, that is, inhibition of PDE4 would increase cAMP and activation of PKA, which would catalyze more extensive phosphorylation of PDE4 to produce higher affinity for certain inhibitors. Positive feedback loops, such as these for PDEs 4 and 5, will clearly impact the range of potency determined for compounds that are currently under development as well as their pharmacokinetic properties.

2.4 Interaction with Other Proteins

For many years, PDE activity in cells was referred to either as soluble or particulate, but in most cases, there has been little understanding of either the importance or the molecular basis for this partitioning. In fact, members of the same PDE families could be found in both the cytosolic and the particulate fractions. Such complexes occur both at particular cell membranes and within the cytosolic fraction. More recently, it has become apparent that one reason for the diversity of PDEs in most tissues is to provide for their selective interaction with other proteins in signaling complexes allowing dynamic regulation of particular signaling processes and thus giving rise to the concept of “targeted cAMP degradation” (Houslay 2001, 2010). Indeed, it was initial studies in cardiac myocytes that provided a major building block for our understanding of compartmentalized cAMP signaling and its mechanistic implications (Corbin et al. 1977; Hayes et al. 1979; Steinberg and Brunton 2001; Zaccolo and Pozzan 2002).

Selective and diverse compartmentalization of a certain portion of the isoforms within a PDE family has now been well described for PDEs 1, 2, 3, 4, 5, 6, and 10. It seems highly likely that interaction of these PDEs with other proteins to form signaling complexes will affect inhibitor potencies. Indeed, this has been clearly demonstrated for various PDE4 partnerships, such as PDE4A4 with various SH3 domain containing proteins (McPhee 1999); PDE4D5 with RACK1 (Yarwood et al. 1999) and PDE4A4 with the aryl hydrocarbon interacting protein (AIP) (Bolger et al. 2003), where recent structural studies have afforded a potential molecular explanation for such actions (Burgin et al. 2010).

In addition, the determinants that dictate the proportion of a PDE family that is localized to particular intracellular domains or “free” in the cytosol are only beginning to be understood (Houslay 2010) and include competition by different scaffolds for binding specific PDEs and dynamic changes in association patterns that are triggered by phosphorylation (Collins et al. 2008; Murdoch et al. 2007) and ubiquitination (Li et al. 2009). Moreover, when “free” PDEs are translocated from the cytosol to a more restricted and defined locale, the impact on the cN metabolism in the cellular territory that was vacated has yet to be formally considered. Are there “pools/banks” of PDEs that are simply not needed in normal cellular functioning and await a call for action as needed in particular locales in the cell? Are these meaningful pools of PDE activity that should/could be targeted by inhibitors? The very different actions of inhibitors selective for different cAMP-hydrolyzing PDEs, such as PDEs1/2/3/4, were originally interpreted as reflecting actions related to the relative abundance of such enzymes in cells. However, it is now clear that functionally distinct pools of cAMP regulated by targeted PDEs is critical in controlling key cellular processes in cells. The most conclusive proof for a specific mechanism of subcellular targeting comes from studies on PDE4s (Houslay 2001, 2010). Critical to this concept was the discovery that PDE4A1, an enzyme that is entirely membrane bound, is generated by splicing a membrane-targeting domain located within its isoform-specific amino-terminal amino acid sequence to a segment of sequence

containing the regulatory and catalytic domains, that on its own is fully soluble, fully active, and common to all members of the PDE4A subfamily (Baillie et al. 2002; Huston et al. 2006; Scotland and Houslay 1995; Shakur et al. 1993; Smith et al. 1996).

Targeting of PDEs to particular regions of the cell is often dependent on interaction with other proteins. Myriad proteins have been identified that interact with the PDE4 family as a whole by binding to sites within core regions that are conserved across the entire family or are specific for particular PDE4s by binding to regions that are only common within specific subfamilies (Houslay 2001, 2010; Houslay et al. 2007). Such targeting allows particular PDE4 isoforms to associate with specific signaling complexes and control the local cAMP level. This targeting of specific PDEs underpins compartmentalized cAMP signaling, controlling the threshold and persistence for activation of PKA and EPAC, for example, within the environs of the complex containing the particular PDE that is sequestered there (see e.g., Huston et al. 2008). Indeed, the use of spatially constrained, genetically encoded cAMP reporters has allowed defined “pools” of cAMP to be detected and the role of certain sequestered PDEs to be shown as pivotal in establishing and maintaining cN gradients in cells (Mongillo et al. 2004, 2006; Penmatsa et al. 2010; Rich et al. 2001a, b, 2006; Zaccolo and Pozzan 2002).

PDE3A has also been shown to specifically form complexes with a variety of proteins including 14-3-3 proteins, plectin, brefeldin A-inhibited guanine nucleotide exchange proteins, and the cystic fibrosis transmembrane conductance regulator channel (CFTR); in some instances, these localizations have also been shown to control local pools of cAMP (Barnes et al. 2005; Mongillo et al. 2004; Penmatsa et al. 2010; Puxeddu et al. 2009; Tasken et al. 2001). Likewise, a portion of platelet PDE5 has been shown to form a complex with PKG and be selectively activated by elevation of cGMP, thereby regulating both local cGMP level and calcium transients (Wilson et al. 2008). The presence of PDE5 in cardiomyocytes is controversial (Lukowski et al. 2010; Vandeput et al. 2009). However, some investigators report it to be present at the z-bands along with PKG, and their results suggest that this localization of PDE5 is required for the antihypertrophic effects of sildenafil in the heart (Kass et al. 2007; Nagayama et al. 2008; Takimoto et al. 2005a, b). PDE7A1 and the C subunit of PKA have been shown to interact with high affinity ($K_D \sim 0.5$ nM), thereby blocking the catalytic activity of the C subunit, but the effect of this interaction on catalytic function/inhibitor sensitivity of the PDE is not known (Han et al. 2006).

Targeting a specific PDE isoform to a particular intracellular locale can be expected to confer a functional role on that isoform that is inherently associated with its unique spatial sequestration (Houslay 2010). This property cannot be gauged by the use of selective inhibitors, genetic ablation (Jin et al. 1999), or siRNA knockdown (Lynch et al. 2005) since each of these approaches will target both the sequestered and free populations of that PDE. Instead, the use of dominant-negative constructs has been successfully exploited to address this problem (Baillie et al. 2003; Lynch et al. 2005; McCahill et al. 2005). In this approach, a single point mutation that ablates catalytic activity while retaining overall structural integrity of the PDE is engineered. Overexpression of such a catalytically inactive PDE

construct in cells displaces the cognate, endogenous, active, and sequestered form from its site of action without affecting the activity of free populations of that PDE. In so doing, local cAMP or cGMP concentration around the complex involving the sequestered PDE will selectively rise, thereby generating a unique phenotypic signature that mimics inhibition of the sequestered PDE. It will be of great interest if either small molecules or peptidomimetics that disrupt specific PDE partnerships can be identified for therapeutic advantage. Certainly proof of principal has been garnered for this approach by the development of dominant negative constructs for PDE4 isoforms as elucidated above (Lynch et al. 2005) and use of cell-permeable peptides that duplicate the binding surface of one partner and thus disrupt specific PDE partnerships in intact cells (Murdoch et al. 2007; Smith et al. 2007).

The concept that PDEs interact with and are regulated by other proteins was first discovered from studies of the PDE1 family, which is regulated by reversible interaction with calmodulin, and the PDE6 family, which is located in photoreceptor rod and cone cells and regulated by interaction with the small inhibitory $P\gamma$ proteins and activated transducin. For other PDE families, appreciation of this type of regulation is relatively new. Consequently, the focus in studying protein interactions involving most PDEs has been devoted to identifying protein partnerships, defining modes of interaction, and appreciating the functional significance of such interactions. The paradigm for PDE partner proteins that profoundly regulate PDE catalytic site functions is well illustrated by control of PDE6 cN-hydrolyzing activity through the direct interaction of its catalytic subunits with its inhibitory $P\gamma$ -subunits and the effect of transducin in the activated state to bind to PDE6 γ in that complex, thereby relieving the PDE6 γ inhibitory effects (Bender and Beavo 2006). Elegant biochemical and structural studies have recently allowed the molecular basis of this to be determined (Barren et al. 2009; Zhang et al. 2009). Two distinct types of interactions between $P\gamma$ and PDE6 catalytic subunits that provide for the potent inhibition of PDE6 catalysis have been proposed. One set of interactions involves direct contact of the carboxyl-terminal residues of $P\gamma$ with the PDE6 catalytic pocket, thereby blocking cGMP entry. The second set of interactions involves binding of other regions of $P\gamma$ to the PDE6 catalytic subunit, so as to attenuate catalytic activity in an allosteric manner. Thus, activation of PDE6 by the GTP-bound form of transducin apparently requires interaction with the carboxyl-terminus of PDE6 γ as well as additional regions of PDE6 γ to relieve the inhibitory constraint on the PDE6 catalytic subunits. Proteins that interact with members of other PDE families may also employ the strategy of multiple contacts that diversely influence enzymatic activity, specific localization, and functional features; such complexities should always be considered.

The regulation of PDEs by protein–protein interactions has been explicitly and elegantly addressed in studies performed with PDE4, where a large number of binding partners have been identified (Houslay 2010). It is already appreciated from phosphorylation studies of PDE4 isoforms (described above) that conformational changes induced by phosphorylation are associated with altered activity and sensitivity to some inhibitors (Conti et al. 2003; Houslay and Adams 2003; Houslay et al. 2005). Four PDE4 genes (A/B/C/D) encode around 25 isoforms that are characterized by isoform-specific amino-terminal regions. These are then grouped based

upon the presence of regulatory UCR domains (UCR1 and UCR2), with long forms having both UCRs, short forms having UCR2, super-short forms having a truncated UCR2 and the catalytically inactive, dead-short isoforms having neither (Bolger et al. 2006; Conti and Beavo 2007; Houslay 2001, 2010).

While the structure of the catalytic domains from all four PDE4 subfamilies is known (Ke and Wang 2007a), until very recently nothing has been known about the structure of the UCR regulatory domains or interactions of UCRs with the catalytic domain. A recent report documents the X-ray crystal structure of UCR2, a helical structure that can bind adjacent to and over the PDE4 catalytic pocket, thereby gating cN substrate access (Burgin et al. 2010). This gives a structural basis to the previous biochemical evidence and proposal that UCR2 provides an autoinhibitory domain (Lim et al. 1999) and for previous observations that PDE4 enzymes can adopt distinct conformational states that vary in sensitivity to certain inhibitors (Houslay 2001; Houslay and Adams 2003; Houslay et al. 2005). Burgin et al. used information derived from a collection of X-ray crystal structures of PDE4 isoenzymes, molecular modeling, site-directed mutagenesis, and systematic synthetic chemistry to produce the novel small molecule inhibitors of PDE4D. These are bifunctional compounds that form contacts with both the catalytic site and the UCR2, thereby locking the enzyme in the inactive state; the double set of contacts increases points of interaction with PDE4, thereby enhancing inhibitory potency (Burgin et al. 2010; Houslay and Adams 2010).

The diverse interactions of various inhibitors with PDE4 are exemplified by RS25344 and roflumilast. RS25344 interacts with the gating sequence, which stabilizes the UCR2-capped state (Burgin et al. 2010), whereas roflumilast, which has recently been approved in Europe for treatment of chronic obstructive pulmonary disease (COPD) (see Tenor et al. 2011), occupies the uncapped catalytic pocket and interacts minimally with residues in the UCR. These discoveries provide the beginnings of insight into the complex inhibition kinetics of compounds, such as rolipram, that may have different affinity for the catalytic pocket in the UCR2-uncapped and UCR2-capped states. This discovery also offers a molecular explanation (Burgin et al. 2010) for the reported observations that PKA phosphorylation alters the inhibitor sensitivity (Hoffmann et al. 1998; Sette and Conti 1996a), for example, by stabilizing an uncapped, activated state. Some of the new compounds produce only partial inhibition (80–90%) of catalytic activity, which may contribute to their actions in the biological setting. The proximity and arrangement of regulatory domains of other PDEs with respect to their catalytic sites as well as their mechanisms of activation/autoinhibition will likely dictate whether this innovative approach to drug design is useful for other PDEs or restricted to the PDE4 family.

The recent structural insights for UCR2 interaction with the PDE4 catalytic unit (Burgin et al. 2010) will undoubtedly stimulate new lines of research and drug discovery relating to PDE4. However, it is also likely to provide a stimulus to understanding of the PDE superfamily as a whole, where paired regulatory regions, for example, GAFs, located amino-terminal to the catalytic domain are common. Solution of the X-ray crystallographic structure of the PDE2 holoenzyme has suggested a different mechanism of regulation of catalytic activity (Pandit et al.

2009). Thus, questions still awaiting an answer include whether distinct mechanisms of regulation of the catalytic domain are present in PDEs, how these interactions might be altered by either protein–protein interaction, phosphorylation, or other modifications, the physiological significance of this and how this all might be exploited to generate novel therapeutics. Also, despite the historical importance of PDE1 in understanding calcium/calmodulin activation of cAMP and cGMP degradation (Bender and Beavo 2006; Goraya and Cooper 2005), there are still no structural insights into the mechanisms whereby the calcium/calmodulin complex interacts with the paired binding domains in PDE1 to elicit activation of this enzyme.

2.5 Exposure to Drugs

PDEs 5 and 4 provide two examples of PDEs that exhibit different affinities for family-selective inhibitors that are impacted by the history of exposure of these proteins to inhibitors.

PDE5 holoenzyme exhibits biphasic affinity for interaction with vardenafil, sildenafil, and tadalafil, that is, a low-affinity site and a high-affinity site, although the inhibitors interact only with the PDE5 catalytic site. Prolonged exposure of PDE5 holoenzyme to these inhibitors converts the kinetics of the low-affinity interaction to that of the high-affinity site (Fig. 5) (Blount et al. 2007; Rybalkina et al. 2010). This shift in affinity for the inhibitors indicates a functional transition that is associated with a physical change in the protein following inhibitor binding. It does not occur in the isolated catalytic domain, but when GAF-B is appended to the catalytic domain, the shift in affinity is like that observed in the holoenzyme. Thus, the affinity of PDE5 for these inhibitors is mediated by cGMP-dependent and cGMP-independent mechanisms, the latter being an effect of the inhibitor itself. Recently, it has been suggested that aggregation of PDE5 fostered by high concentrations of the enzyme in particular tissues may also impact affinity for certain inhibitors (Rybalkina et al. 2010). These observations emphasize once again the importance of not only examining inhibitor potencies within the context of PDE holoenzymes but also with consideration of the molecular status and concentration of the PDE within local environs in the cell.

Interaction of PDE4 isoenzymes with the inhibitor rolipram and related compounds (such as RS-25344) has also uncovered both high- and low-affinity states (Alvarez et al. 1995; Souness and Rao 1997). It should be noted that this is not a generalized property, as inhibitors derived from other classes of compounds, for example, piclamilast, do not share this property and behave as simple competitive inhibitors of PDE4s (Tian et al. 1998; Zhao et al. 2003). This complexity of rolipram interactions with PDE4s was originally uncovered by measurements of isotopically labeled rolipram binding to the two conformation states termed *High-Affinity Rolipram-Binding Sites* (HARBS) and *Low-Affinity Rolipram-Binding Sites* (LARBS) (Houslay and Adams 2003; Jacobitz et al. 1996; Rocque et al. 1997; Souness and Rao 1997; Tian et al. 1998). Both have been thought to reflect

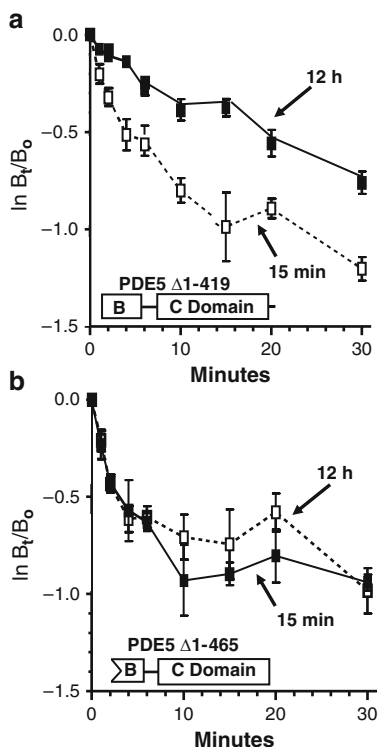


Fig. 5 Exchange-dissociation of [³H]tadalafil from PDE5 $\Delta 1-419$ but not PDE5 $\Delta 1-465$ is affected by time of preincubation with the inhibitor. PDE5 $\Delta 1-419$ (*top*) or PDE5 $\Delta 1-465$ (*bottom*) (80 μ l; 0.035 nM final) was added to 4.5 ml of binding reaction mixture containing [³H]tadalafil (30 nM final concentration) after being preincubated for either 15 min or 12 h at 4°C. The zero time point (B_0) was determined as described in Blount et al. (2007). Following 15-min or 12-h preincubation of the respective constructs with [³H]tadalafil, the stoichiometry of inhibitor binding (B_0) was not significantly different. Approximately 30 μ l of a 1 mM tadalafil solution was then added to the remaining incubating binding reaction mixture at 4°C. Aliquots were removed at various times (B_t) and filtered by the same procedure at the indicated time points

characteristics of apparently different conformations of the same catalytic site; however, results of recent X-ray crystal structures of various constructs of PDE4 isoforms in complex with certain inhibitors (described above) reveal that the high-affinity interaction of some inhibitors results from direct contacts with the catalytic pocket as well as with UCR2 (Burgin et al. 2010; Houslay and Adams 2010).

The molecular factors that determine whether the HARBS or LARBS form predominates for a particular inhibitor have not been unequivocally identified, nor has it been established whether these states are interconvertible, as occurs when inhibitor binding to the PDE5 catalytic site shifts the low-affinity site to the high-affinity site. The chemical characteristics of the inhibitor, the contacts that it forms with the catalytic site and/or other regions of the PDE, as well as the oligomerization state, degree of phosphorylation, or metal-ion occupancy of the catalytic center

have all been implicated in stabilizing these dual states (Laliberte et al. 2000). A consistent finding is that the high-affinity conformation is usually reduced in truncated PDE4 enzymes, indicating that the amino terminus of PDE4 exerts important constraints on the conformation of the catalytic domain (Conti et al. 2003). Whether these sites can be flexibly interconverted or are “fixed” in perpetuity will impact considerations regarding future design of PDE inhibitors targeting these PDEs. The dual-affinity state of PDE4s has been used in the PDE4 field as a guiding criterion to predict therapeutic windows of PDE4 inhibitors under development with respect to undesirable side effects of PDE4 inhibitors that have been associated with HARBS. It seems likely that isoforms of other PDE families will also have multiple conformations that exhibit varied affinities for particular inhibitors.

3 Design of PDE Inhibitors

3.1 Inhibitor Design

Emerging appreciation of the many factors that contribute to the physiologically relevant action of PDEs has expanded options for development of new inhibitors. Medicinal chemists are now setting goals for design of a spectrum of inhibitors that selectively target either particular PDE catalytic sites, individual allosteric sites such as the GAFs in PDEs 2, 5, 6, 10, or 11, UCRs in PDE4, a combination of the catalytic sites and regulatory domains, or sites that provide for PDE interactions with proteins/lipids to localize the PDE to particular regions of the cell (Burgin et al. 2010; Keravis and Lugnier 2010; Verhoest et al. 2009). Even with the availability of a number of clinically approved inhibitors that are selective and potent for a particular PDE family, for example, PDE5, development of a different class of inhibitors may be needed to address specific pharmacokinetic needs in optimizing use in diverse medical regimens, for example, compounds with improved bioavailability, slowed clearance, improved stability, brain penetration, etc. (Owen et al. 2009). Moreover, new classes of inhibitors that block only a portion of a particular PDE activity may have merit that has not been appreciated previously (Burgin et al. 2010).

Traditionally, medicinal chemists have generated compounds that directly compete with cN substrate for access to the catalytic sites of PDEs. This has primarily been achieved by systematically modifying a chemical scaffold derived from compounds known to interact with or inhibit PDE catalytic activity. The trial-and-error approach using myriad derivatives of a lead scaffold has successfully produced numerous potent and selective PDE inhibitors with diverse structural characteristics; many of these compounds incorporate the purine of the cNs as the basic scaffold with the goal of developing inhibitors that are substrate mimics but that also include additional elements to enhance affinity and selectivity for a particular group of PDEs (Fig. 6). However, selective inhibitors for most PDE families are still not commercially available despite the great need for their use in biochemical investigations and clinical settings.

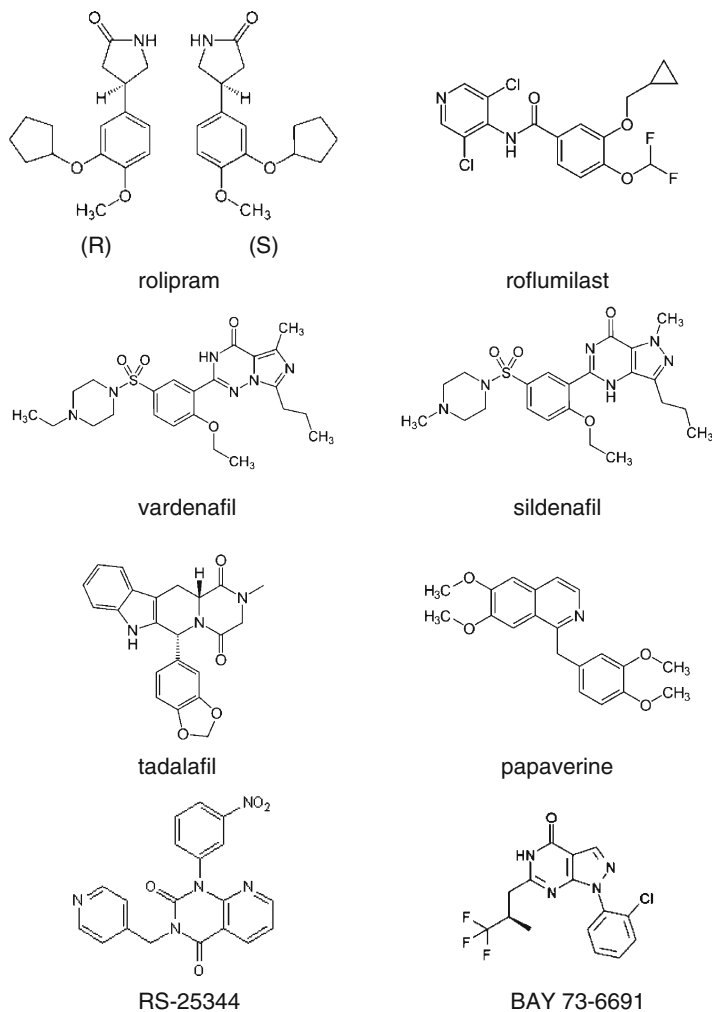


Fig. 6 Structure of the most common nonselective or family-specific PDE inhibitors. Rolipram, Roflumilast and RS25344 are PDE4-selective. The two enantiomers of rolipram [(*R*)-(–)-rolipram (the more potent enantiomer of rolipram) and (*S*)-(+)-rolipram (the less potent enantiomer of rolipram)] are indicated as (*R*) and (*S*), respectively. Vardenafil, sildenafil, and tadalafil are PDE5-selective, although vardenafil and sildenafil are potent inhibitors of PDE6. BAY 73-6691 is selective for PDE9. Papaverine is nonselective but has high affinity for PDE10

Guided by insights derived from X-ray crystal structures of various PDE catalytic sites as well as GAFs and UCR domains, chemists are now systematically exploiting features that provide for novel drug designs exhibiting greater specificity and potency. Structural information that is available for many isolated catalytic domains reveals new insights into specific topographical features of particular catalytic sites. Much effort is now being devoted to utilization of this new information for

development of more selective inhibitors, for example, development of inhibitors that selectively target the catalytic sites of the respective PDE4 isoforms, which have clear clinical implications. Although this approach has been slow to produce better inhibitors, some progress is being made as evidenced by the recent emergence of a specific, a potent PDE10 inhibitor (Verhoest et al. 2009), an improved PDE5 inhibitor (Owen et al. 2009), and new PDE4 inhibitors (Burgin et al. 2010).

3.2 Inhibitor Selectivity and Potency

The goals involved in generating desirable PDE inhibitors include selectivity and potency. Selectivity is a key feature in most instances; typically, a compound that exhibits potency for one PDE that is 50- to 100-fold greater than potency of inhibition for other PDEs is deemed to be a “specific” inhibitor. However, this measure is not always rigorously applied in the literature. Although sildenafil and vardenafil are commonly referred to as PDE5-selective or PDE5-specific, they also potently inhibit PDE6 (Zhang et al. 2005). This close overlap in function confounds use of these compounds in studies involving visual transduction or pineal functions. The restriction of PDE6 expression to photoreceptor cells and the pineal gland minimizes the issue surrounding this lack of selectivity between these two families, but it should be kept in mind for studies involving neuronal tissues or other tissues, where PDE6 might be expressed (Bazhin et al. 2010). Likewise, even when an inhibitor meets the criteria of being defined as “specific” for one PDE versus another, partial inhibition of the other PDE may come into play in the physiological setting (Lukowski et al. 2010).

Potency, while generally desirable, may have reasonable limitations. A potent inhibitor is likely to require lower doses to achieve the desired effect thus translating into lower drug exposure for the patient and lesser chance for unanticipated reactions. However, to inhibit a meaningful portion of the activity of a PDE in a tissue, there will have to be sufficient circulating inhibitor to block a significant portion of that activity. It has been conjectured that high-affinity interaction between an inhibitor and a PDE could foster increased concentration of the inhibitor in the cell until the PDE is saturated, that is, the PDE would simply soak up the inhibitor as long as high-affinity sites are available (Francis et al. 2008). In these cases, the absolute concentration of the PDE in a particular cell would determine the amount of the high-affinity inhibitor localized to the cellular milieu.

4 Interactions of PDE Inhibitors with Multiple PDEs or Non-PDE Proteins

New drugs are typically designed to selectively interact with one targeted protein, and PDE inhibitors are no exception. However, the molecular scaffold of many PDE inhibitors mimics the purine in cAMP and cGMP. Given the number of

cellular proteins that interact with purine-containing compounds, cross-reaction is always a consideration, and detection of such unintended interactions can be challenging. Moreover, PDE inhibitors whose structures or components of these structures that do not mimic the purine in cNs have also been found to interact with non-PDE cellular proteins. When possible, employing several PDE inhibitors with different structures in a study significantly strengthens the validity of the interpretations. Concepts based on biochemical results associated with use of one inhibitor may need to be revisited if other interactions are subsequently found (Ohshiro et al. 2008; Taniguchi et al. 2006; Thompson 1991).

4.1 Interaction with Multiple PDEs

Recently, consideration has been given to the potential clinical advantages of combining administration of compounds that are selective for different PDEs, utilizing compounds that inhibit two PDEs, that is, dual-selective compounds, or use of more nonselective compounds that inhibit different PDEs (Giembycz 2006). For instance, inhibitors that block either PDE5 or PDE1 appear to impede pathological remodeling in vascular smooth muscle and cardiac tissue, although the relative roles of these PDEs is yet to be determined (Kang et al. 2003; Kass et al. 2007; Kim et al. 2001; Miller et al. 2009; Rybalkin et al. 1997, 2003; Takimoto et al. 2005a; Zhu and Strada 2007). However, the potential to combine selective inhibitors for both PDE families as a therapy to block the remodeling merits further study. The rationale for the use of either dual-selective or nonselective inhibitors is that relatively low concentration of such an inhibitor could partially block several PDEs, for example, PDE5/PDE1, PDE4/PDE1, PDE4/PDE3, etc. in a given tissue, to produce significant increases in cNs. In contrast to the concept described above of nearly absolute specificity of a particular PDE to control cAMP or cGMP in a particular locale, this approach assumes that at least in some situations multiple PDEs work jointly to affect cN concentrations. The hypothesis suggests that moderate blockage of several PDEs might produce modest, but physiologically relevant, changes in cN without incurring the adverse effects commonly associated with more marked changes in cN in either the target or the nontarget tissues when a specific inhibitor more completely blocks a particular PDE (Giembycz 2006). This is conceptually similar to the potential advantages of partial inhibition of a particular PDE described above (Burgin et al. 2010).

4.2 Interaction with Other Proteins in the cN-Signaling Pathway

Use of compounds designed to block PDE action as well as activating other proteins in the cN-signaling pathway is also worthy of consideration. cN analogs commonly function not only as activators of particular cN-dependent protein kinases, cN-gated channels, or EPACs, but also as inhibitors of certain PDEs; many cN analogs are

highly resistant to breakdown by PDEs (Beltman et al. 1995; Butt et al. 1995; Poppe et al. 2008; Sekhar et al. 1996). Low doses of such compounds would be predicted to work on two target proteins to synergistically foster increased cN signaling through that pathway. Examples include YC-1 and BAY 41-2272, both of which are NO-independent activators of soluble guanylyl cyclase and act to elevate cGMP in platelets, inhibit platelet aggregation, and lower blood pressure (Friebe et al. 1998; Galle et al. 1999; Mullershausen et al. 2004; Stasch and Hobbs 2009); each also acts as a PDE inhibitor. Thus, the biological actions result from the simultaneous increase in cGMP production and inhibition of cGMP breakdown. The combined effects of such compounds at two points in a pathway would foster a synergistic increase in cN signaling and potentially produce meaningful changes in signaling even at low ligand concentrations.

4.3 Interaction with Other Proteins

Interaction of PDE inhibitors with proteins other than PDEs is a serious problem that is quite commonly ignored by investigators. However, this oversight compromises many studies since many investigators accept the hyperbole asserting that compounds are truly “specific and selective” for the targeted PDE.

The use of zaprinast, which has long been touted as a selective inhibitor for PDE5/PDE6, is a prime example of such a problem. The interpretation of the results of the vast majority of studies where zaprinast has been used attributes its effects entirely to inhibition of PDE5. Its action to also inhibit PDE1 with reasonable potency is largely ignored. However, zaprinast has recently been shown to be a potent agonist for GPR35 (EC_{50} as low as 16 nM), a widely expressed receptor for kynurenic acid (Ohshiro et al. 2008; Taniguchi et al. 2006). In contrast to the widely and erroneously accepted action of zaprinast to exclusively inhibit PDE5 ($IC_{50} \sim 130$ nM), promote elevation of cNs and lower calcium, its action on GPR35 inhibits adenylyl cyclase activity and induces intracellular calcium mobilization. Notably, these effects are not mimicked by two potent PDE5/PDE6 inhibitors that structurally differ from zaprinast. In referring to the earlier studies, the authors of the report note “it may be necessary to repeat those experiments (*done only with zaprinast*) with different structural classes of selective PDE inhibitors.”

Theophylline, a natural compound and one of the first PDE inhibitors to be discovered, is still in wide use for investigational purposes and various clinical treatments; its biological effects are mediated by actions as an antagonist of A_1 -adenosine receptors or as a nonspecific inhibitor of PDEs. Relatively high plasma concentrations of theophylline (10–20 mg/L, which translates to ~50–100 μ M) are required to elicit significant bronchodilation (Barnes 2006). Based on IC_{50} values of theophylline for several PDEs, it is entirely possible that this level could significantly diminish PDE activity, thereby promoting cN elevation (Butcher and Sutherland 1962; Dent and Rabe 1996), and it is well accepted that the relationship between the extracellular concentration of a PDE inhibitor and its intracellular

concentration cannot be accurately predicted (Thompson 1991). Evidence derived from studies of airway smooth muscle indicates that effects of theophylline in that tissue are mediated through inhibition of PDEs 3–5 to cause increases in cAMP and cGMP and activation of the respective signaling pathways (Cortijo et al. 1993; Rabe et al. 1995). Effects of theophylline to blunt airway inflammation in COPD occur below 10 mg/ml plasma (Hirano et al. 2006; Kobayashi et al. 2004) and are therefore unlikely to act through PDE inhibition.

Cilostazol is somewhat selective as an inhibitor of the PDE3 family ($IC_{50} \sim 0.5 \mu\text{M}$) and is marketed for treatment of intermittent claudication (Kambayashi et al. 2003); it also reportedly inhibits PDE5 with nine-fold lower affinity ($IC_{50} \sim 4.4 \mu\text{M}$) (Sudo et al. 2000) and antagonizes adenosine uptake at clinically relevant concentrations (3 μM). The inhibitory effect of cilostazol for both PDE3 and adenosine uptake was not appreciated initially, but now this dual effect is suggested to be a significant factor in the overall safety and efficacy of cilostazol compared to PDE3 inhibitors that do not block adenosine uptake (Kambayashi et al. 2006). Since the clinically relevant concentration of cilostazol is also in the range of its IC_{50} for PDE5, it is plausible that inhibition of PDE5 may account for a portion of cilostazol's vasodilatory and antiaggregatory effects on vascular smooth muscle and platelets, respectively, where PDE5 is abundant.

Dipyridamole, a PDE inhibitor that is approved for clinical use for prevention of ischemic events following stroke, also has dual actions; it is a somewhat nonselective inhibitor of PDEs (PDE5 $IC_{50} \sim 0.9 \mu\text{M}$, PDE7 $IC_{50} \sim 0.6 \mu\text{M}$, PDE8 $IC_{50} \sim 9 \mu\text{M}$, PDE10 $IC_{50} \sim 1.0 \mu\text{M}$, and PDE11 $IC_{50} \sim 0.4 \mu\text{M}$) (Beavo and Brunton 2002) and a relatively potent inhibitor of adenosine uptake (Klabunde 1983; Schaper 2005). Although the antiaggregatory effects of dipyridamole in platelets are largely attributed to its action to block adenosine uptake, its concentration in plasma (1.7 $\mu\text{g/L}$ or $\sim 3.5 \mu\text{M}$) is sufficient to block PDE5 as well (Serebruanu et al. 2009); the combined actions of this drug would decrease platelet aggregation.

Although the effects of the PDE5 inhibitors, vardenafil, sildenafil, and tadalafil are thought to be mediated exclusively through inhibition of PDE5, differences in the effects of vardenafil versus those of sildenafil and tadalafil in rabbit pulmonary arteries and human platelets have been reported (Toque et al. 2008). The evidence in this report suggests that vardenafil, in addition to acting as a PDE5 inhibitor in these tissues, may also block store-operated calcium channels. This possibility warrants further study. A recent report indicates that *in vitro* sildenafil activates several carbonic anhydrase isoenzymes (hCA I, hCA VB and hCA VI) with K_a values in the range of 1–7 μM (Abdulkadir Coban et al. 2009); this effect may be mediated by the piperazine moiety in sildenafil since other carbonic anhydrases activators contain a piperazine moiety. Whether this effect impacts the pharmacological actions of sildenafil is not known, but since plasma sildenafil (bound and free) in a typical patient approaches 1 μM , the possibility should not be entirely dismissed. This effect emphasizes that a variety of molecular features of PDE inhibitors may contribute to unanticipated interactions. Recently, Jeon et al. reported that the anti-inflammatory action of vinpocetine, which has traditionally been considered to be a PDE1-selective inhibitor, proceeds through a PDE-independent

pathway (Jeon et al. 2010). Finally, EHNA, a selective and weak inhibitor of PDE2, also inhibits adenosine deaminase (Mery et al. 1995), which complicates its use in biochemical studies.

5 Concluding Remarks

Major advances have been made in recent years in defining the physical and chemical characteristics of the catalytic sites of PDEs and identifying improved strategies for development of potent and selective inhibitors of the various PDE families. X-ray crystallographic structures of the catalytic domains of numerous PDEs has allowed for precise spatial considerations for future design of more selective inhibitors. More recently, the X-ray crystal structures of regulatory domains of these proteins have become available and provide new opportunities for development of PDE inhibitors. The clinical success of the PDE5 inhibitors has been a major advance in understanding the medical implications of these types of drugs. Despite the availability of some selective PDE inhibitors, it is clear that great caution is warranted when using these compounds. There are now well-documented examples in which a PDE inhibitor that was considered to specifically target a particular PDE has mechanisms of action that are unrelated to inhibition of PDE activity. In addition, interpretation of results based on the concentration of a “selective” inhibitor that is applied extracellularly is particularly problematic since it is not possible to discern the concentration of that compound in the intracellular milieu or in particular cellular compartments where it might reach concentrations sufficient for inhibition of other PDEs. Use of several inhibitors and a variety of approaches is important to validate interpretation of results in such studies. Innovative strategies are needed to generate new inhibitors that are selective for specific PDE families and their subfamilies since these compounds are greatly needed for investigational purposes that will hopefully lead to clinical use.

References

- Abdulkadir Coban T, Beydemir S, Gucin I, Ekinci D, Innocenti A, Vullo D, Supuran CT (2009) Sildenafil is a strong activator of mammalian carbonic anhydrase isoforms I–XIV. *Bioorg Med Chem* 17:5791–5795
- Alvarez R, Sette C, Yang D, Eglén R, Wilhelm R, Shelton ER, Conti M (1995) Activation and selective inhibition of cyclic AMP-specific phosphodiesterase, PDE4D3. *Mol Pharmacol* 48:616–622
- Aravind L, Ponting CP (1997) The GAF domain: an evolutionary link between diverse phototransducing proteins. *Trends Biochem Sci* 22:458–459
- Baillie GS, Huston E, Scotland G, Hodgkin M, Gall I, Peden AH, MacKenzie C, Houslay ES, Currie R, Pettitt R, Walmsley AR, Wakelam MJO, Warwicker J, Houslay MD (2002) TAPAS-1, a novel microdomain within the unique N-terminal region of the PDE4A1 cAMP specific phosphodiesterase that allows rapid, Ca^{2+} -triggered membrane association with selectivity for interaction with phosphatidic acid. *J Biol Chem* 277:28298–28309

- Baillie GS, Sood A, McPhee I, Gall I, Perry SJ, Lefkowitz RJ, Houslay MD (2003) β -arrestin mediated PDE4 cAMP phosphodiesterase recruitment regulates β_2 -adrenoceptor switching from G_s to G_i . *Proc Natl Acad Sci* 100:940–945
- Barnes P (2006) Theophylline for COPD. *Thorax* 61:742–744
- Barnes AP, Livera G, Huang P, Sun C, O'Neal WK, Conti M, Stutts MJ, Milgram SL (2005) Phosphodiesterase 4D forms a cAMP diffusion barrier at the apical membrane of the airway epithelium. *J Biol Chem* 280:7997–8003
- Barren B, Gakhar L, Muradov H, Boyd KK, Ramaswamy S, Artemyev NO (2009) Structural basis of phosphodiesterase 6 inhibition by the C-terminal region of the gamma-subunit. *EMBO J* 28:3613–3622
- Bazhin AV, Tambor V, Dikov B, Philippov PP, Schadendorf D, Eichmuller SB (2010) cGMP-phosphodiesterase 6, transducin and Wnt5a/Frizzled-2-signaling control cGMP and Ca(2+) homeostasis in melanoma cells. *Cell Mol Life Sci* 67:817–828
- Beavo JA, Brunton LL (2002) Cyclic nucleotide research - still expanding after half a century. *Nat Rev Mol Cell Biol* 3:710–718
- Beltman J, Becker DE, Butt E, Jensen GS, Rybalkin SD, Jastorff B, Beavo JA (1995) Characterization of cyclic nucleotide phosphodiesterases with cyclic GMP analogs: topology of the catalytic domains. *Mol Pharmacol* 47:330–339
- Bender AT, Beavo J (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Bessay E, Blount M, Zoraghi R, Beasley A, Grimes K, Francis S, Corbin JD (2008) Phosphorylation increases affinity of the phosphodiesterase-5 catalytic site for tadalafil. *J Pharmacol Exp Ther* 325:62–68
- Blount MA, Beasley A, Zoraghi R, Sekhar KR, Bessay EP, Francis SH, Corbin JD (2004) Binding of tritiated sildenafil, tadalafil, or vardenafil to the phosphodiesterase-5 catalytic site displays potency, specificity, heterogeneity, and cGMP stimulation. *Mol Pharmacol* 66:144–152
- Blount MA, Zoraghi R, Ke H, Bessay EP, Corbin JD, Francis SH (2006) A 46-amino acid segment in phosphodiesterase-5 GAF-B domain provides for high vardenafil potency over sildenafil and tadalafil and is involved in phosphodiesterase-5 dimerization. *Mol Pharmacol* 70:1822–1831
- Blount MA, Zoraghi R, Bessay EP, Beasley A, Francis SH, Corbin JD (2007) Conversion of phosphodiesterase-5 (PDE5) catalytic site to higher affinity by PDE5 inhibitors. *J Pharmacol Exp Ther* 323:730–737
- Bolger G, Michaeli T, Martins T, St John T, Steiner B, Rodgers L, Riggs M, Wigler M, Ferguson K (1993) A family of human phosphodiesterases homologous to the dunce learning and memory gene product of *Drosophila melanogaster* are potential targets for antidepressant drugs. *Mol Cell Biol* 13:6558–6571
- Bolger GB, Peden AH, Steele MR, MacKenzie C, McEwan DG, Wallace DA, Huston E, Baillie GS, Houslay MD (2003) Attenuation of the activity of the cAMP-specific phosphodiesterase PDE4A5 by interaction with the immunophilin XAP2. *J Biol Chem* 278:33351–33363
- Bolger GB, Conti M, Houslay MD (2006) Cellular functions of phosphodiesterase-4 enzymes. In: Beavo J, Francis S, Houslay MD (eds) *Phosphodiesterases in health and disease*. CRC, Boca Raton, pp 99–130, Ch. 6
- Bos JL (2006) Epac proteins: multi-purpose cAMP targets. *Trends Biochem Sci* 31:680–686
- Braumann T, Erneux C, Petridis G, Stohrer WD, Jastorff B (1986) Hydrolysis of cyclic nucleotides by a purified cGMP-stimulated phosphodiesterase: structural requirements for hydrolysis. *Biochim Biophys Acta* 871:199–206
- Burgers PM, Eckstein F (1979) Stereochemistry of internucleotide bond formation by polynucleotide phosphorylase from *Micrococcus luteus*. *Biochemistry* 18:450–454
- Burgin AB, Magnusson OT, Singh J, Witte P, Staker BL, Bjornsson JM, Thorsteinsdottir M, Hrafnisdottir S, Hagen T, Kiselyov AS, Stewart LJ, Gurney ME (2010) Design of phosphodiesterase 4D (PDE4D) allosteric modulators for enhancing cognition with improved safety. *Nat Biotechnol* 28:63–70

- Butcher RW, Sutherland EW (1962) Adenosine 3', 5'-phosphate in biological materials. I. Purification and properties of cyclic 3', 5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3', 5'-phosphate in human urine. *J Biol Chem* 237:1244–1250
- Butt E, Beltman J, Becker DE, Jensen GS, Rybalkin SD, Jastorff B, Beavo JA (1995) Characterization of cyclic nucleotide phosphodiesterases with cyclic AMP analogs: topology of the catalytic sites and comparison with other cyclic AMP-binding proteins. *Mol Pharmacol* 47:340–347
- Castro LR, Verde I, Cooper DM, Fischmeister R (2006) Cyclic guanosine monophosphate compartmentation in rat cardiac myocytes. *Circulation* 113:2221–2228
- Charbonneau H (1990) Structure-function relationships among cyclic nucleotide phosphodiesterases. In: Beavo J, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases: structure, regulation and drug action*. Wiley, New York, pp 267–296
- Chen G, Wang H, Robinson H, Cai J, Wan Y, Ke H (2008) An insight into the pharmacophores of phosphodiesterase-5 inhibitors from synthetic and crystal structural studies. *Biochem Pharmacol* 75:1717–1728
- Cheung PP, Yu L, Zhang H, Colman RW (1998) Partial characterization of the active site human platelet cAMP phosphodiesterase, PDE3A, by site-directed mutagenesis. *Arch Biochem Biophys* 360:99–104
- Collins DM, Murdoch H, Dunlop AJ, Charych E, Baillie GS, Herberg FW, Brandon N, Prinz A, Houslay MD (2008) Ndel1 alters its conformation by sequestering cAMP-specific phosphodiesterase-4D3 (PDE4D3) in a manner that is dynamically regulated through Protein Kinase A (PKA). *Cell Signal* 20:2356–2369
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C (2003) Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J Biol Chem* 278:5493–5496
- Corbin JD, Francis SH (1999) Cyclic GMP phosphodiesterase-5: target of sildenafil. *J Biol Chem* 274:13729–13732
- Corbin JD, Sugden PH, Lincoln TM, Keely SL (1977) Compartmentalization of adenosine 3':5'-monophosphate and adenosine 3':5'-monophosphate-dependent protein kinase in heart tissue. *J Biol Chem* 252:3854–3861
- Corbin JD, Turko IV, Beasley A, Francis SH (2000) Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. *Eur J Biochem* 267:2760–2767
- Corbin JD, Blount MA, Weeks JL 2nd, Beasley A, Kuhn KP, Ho YS, Saidi LF, Hurley JH, Kotera J, Francis SH (2003) [3H]sildenafil binding to phosphodiesterase-5 is specific, kinetically heterogeneous, and stimulated by cGMP. *Mol Pharmacol* 63:1364–1372
- Cortijo J, Bou J, Beleta J, Cardelus I, Llenas J, Morcillo E, Gristwood RW (1993) Investigation into the role of phosphodiesterase IV in bronchorelaxation, including studies with human bronchus. *Br J Pharmacol* 108:562–568
- Cote RH (2006) Photoreceptor phosphodiesterase (PDE6): a G-protein-activated PDE regulating visual excitation in rod and cone photoreceptor cells. In: Beavo J, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, pp 165–193
- Dent G, Rabe K (1996) Effects of theophylline and non-selective xanthine derivatives on PDE isoenzymes and cellular function. In: Schudt C, Dent G, Rabe KF (eds) *Handbook of immunopharmacology: phosphodiesterase inhibitors*. Academic, San Diego, pp 41–64
- Erneux C, Miot F, Boeynaems JM, Dumont JE (1982) Paradoxical stimulation by 1-methyl-3-isobutylxanthine of rat liver cyclic AMP phosphodiesterase activity. *FEBS Lett* 142:251–254
- Fink TL, Francis SH, Beasley A, Grimes KA, Corbin JD (1999) Expression of an active, monomeric catalytic domain of the cGMP-binding cGMP-specific phosphodiesterase (PDE5). *J Biol Chem* 274:34613–34620
- Francis SH, Corbin JD (1999) Cyclic nucleotide-dependent protein kinases: intracellular receptors for cAMP and cGMP action. *Crit Rev Clin Lab Sci* 36:275–328

- Francis SH, Corbin JD (2005) Phosphodiesterase-5 inhibition: the molecular biology of erectile function and dysfunction. *Urol Clin North Am* 32:419–429, vi
- Francis SH, Corbin JD (2009) Phosphodiesterase-5. In: Bradshaw RA, Dennis EA (eds) *Handbook of cell signaling*, vol 2, 2nd edn. Academic, Oxford, pp 1439–1444
- Francis SH, Colbran JL, McAllister-Lucas LM, Corbin JD (1994) Zinc interactions and conserved motifs of the cGMP-binding cGMP-specific phosphodiesterase suggest that it is a zinc hydro-lase. *J Biol Chem* 269:22477–22480
- Francis SH, Zoraghi R, Kotera J, Ke H, Bessay EP, Blount MA, Corbin JD (2006) Phosphodies-terase-5: molecular characteristics relating to structure, function, and regulation. In: Beavo SHF JA, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, pp 131–164
- Francis SH, Morris GZ, Corbin JD (2008) Molecular mechanisms that could contribute to prolonged effectiveness of PDE5 inhibitors to improve erectile function. *Int J Impot Res* 20: 333–342
- Friebe A, Mullershausen F, Smolenski A, Walter U, Schultz G, Koesling D (1998) YC-1 potentiates nitric oxide- and carbon monoxide-induced cyclic GMP effects in human platelets. *Mol Pharmacol* 54:962–967
- Galle J, Zabel U, Hubner U, Hatzelmann A, Wagner B, Wanner C, Schmidt HH (1999) Effects of the soluble guanylyl cyclase activator, YC-1, on vascular tone, cyclic GMP levels and phosphodiesterase activity. *Br J Pharmacol* 127:195–203
- Giembycz MA (2006) Reinventing the wheel: nonselective phosphodiesterase inhibitors for chronic inflammatory diseases. In: Beavo JA, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, pp 649–665
- Gopal VK, Francis SH, Corbin JD (2001) Allosteric sites of phosphodiesterase-5 (PDE5). A potential role in negative feedback regulation of cGMP signaling in corpus cavernosum. *Eur J Biochem* 268:3304–3312
- Goraya TA, Cooper DM (2005) Ca²⁺-calmodulin-dependent phosphodiesterase (PDE1): current perspectives. *Cell Signal* 17:789–797
- Gross-Langenhoff M, Hofbauer K, Weber J, Schultz A, Schultz JE (2006) cAMP is a ligand for the tandem GAF domain of human phosphodiesterase 10 and cGMP for the tandem GAF domain of phosphodiesterase 11. *J Biol Chem* 281:2841–2846
- Gurney ME, Burgin AB, Magnusson OT, Stewart LJ (2011) Small molecule allosteric modulators of phosphodiesterase 4. Francis SH, Conti M, Houslay MD (eds) *Phosphodiesterases as drug targets*. Springer, Heidelberg
- Han P, Sonati P, Rubin C, Michaeli T (2006) PDE7A1, a cAMP-specific phosphodiesterase, inhibits cAMP-dependent protein kinase by a direct interaction with C. *J Biol Chem* 281:15050–15057
- Handa N, Mizohata E, Kishishita S, Toyama M, Morita S, Uchikubo-Kamo T, Akasaka R, Omori K, Kotera J, Terada T, Shirouzu M, Yokoyama S (2008) Crystal structure of the GAF-B domain from human phosphodiesterase 10A complexed with its ligand, cAMP. *J Biol Chem* 283:19657–19664
- Hayes JS, Brunton LL, Brown JH, Reese JB, Mayer SE (1979) Hormonally specific expression of cardiac protein kinase activity. *Proc Natl Acad Sci USA* 76:1570–1574
- He F, Seryshev AB, Cowan CW, Wensel TG (2000) Multiple zinc binding sites in retinal rod cGMP phosphodiesterase, PDE6alpha beta. *J Biol Chem* 275:20572–20577
- Heikaus CC, Stout JR, Sekharan MR, Eakin CM, Rajagopal P, Brzovic PS, Beavo JA, Klevit RE (2008) Solution structure of the cGMP binding GAF domain from phosphodiesterase 5: insights into nucleotide specificity, dimerization, and cGMP-dependent conformational change. *J Biol Chem* 283:22749–22759
- Hirano T, Yamagata T, Gohda M, Yamagata Y, Ichikawa T, Yanagisawa S, Ueshima K, Akamatsu K, Nakanishi M, Matsunaga K, Minakata Y, Ichinose M (2006) Inhibition of reactive nitrogen species production in COPD airways: comparison between inhaled corticosteroid and oral theophylline. *Thorax* 61:761–766
- Hoffmann R, Wilkinson IR, McCallum JF, Engels P, Houslay MD (1998) cAMP-specific phos-phodiesterase HSPDE4D3 mutants which mimic activation and changes in rolipram inhibition

- triggered by protein kinase A phosphorylation of Ser-54: generation of a molecular model. *Biochem J* 333:139–149
- Houslay MD (2001) Intracellular targeting and regulation of PDE4 cAMP specific Phosphodiesterases. *Prog Nucleic Acid Res Mol Biol* 69:249–315
- Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 35:91–100
- Houslay MD, Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J* 370:1–18
- Houslay MD, Adams DR (2010) Lockdown on phosphodiesterase-4. *Nat Biotechnol* 28:38–40
- Houslay MD, Schafer P, Zhang K (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10:1503–1519
- Houslay MD, Baillie GS, Maurice DH (2007) cAMP-specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signalling. *Circ Res* 100:950–966
- Huai Q, Wang H, Sun Y, Kim HY, Liu Y, Ke H (2003) Three-dimensional structures of PDE4D in complex with roliprams and implication on inhibitor selectivity. *Structure* 11:865–873
- Huai Q, Wang H, Zhang W, Colman RW, Robinson H, Ke H (2004) Crystal structure of phosphodiesterase 9 shows orientation variation of inhibitor 3-isobutyl-1-methylxanthine binding. *Proc Natl Acad Sci USA* 101(26):9624–9629
- Huang D, Hinds TR, Martinez SE, Doneanu C, Beavo JA (2004) Molecular determinants of cGMP binding to chicken cone photoreceptor phosphodiesterase. *J Biol Chem* 279:48143–48151
- Huston E, Pooley L, Julien P, Scotland G, McPhee I, Sullivan M, Bolger G, Houslay MD (1996) The human cyclic AMP-specific phosphodiesterase PDE-46 (HSPDE4A4B) expressed in transfected COS7 cells occurs as both particulate and cytosolic species that exhibit distinct kinetics of inhibition by the antidepressant rolipram. *J Biol Chem* 271:31334–31344
- Huston E, Houslay TM, Baillie GS, Houslay MD (2006) cAMP phosphodiesterase-4A1 (PDE4A1) has provided the paradigm for the intracellular targeting of phosphodiesterases, a process that underpins compartmentalized cAMP signalling. *Biochem Soc Trans* 34:504–509
- Huston E, Lynch M, Mohamed A, Collins DM, Hill EV, MacLeod R, Krause E, Baillie GS, Houslay MD (2008) EPAC and PKA allow cAMP dual control over DNA-PK nuclear translocation. *Proc Natl Acad Sci USA* 105:12791–12796
- Jacobitz S, McLaughlin MM, Livi GP, Burman M, Torphy TJ (1996) Mapping the functional domains of human recombinant phosphodiesterase 4A: structural requirements for catalytic activity and rolipram binding. *Mol Pharmacol* 50:891–899
- Jarvest RL, Lowe G, Baraniak J, Stec WJ (1982) A stereochemical investigation of the hydrolysis of cyclic AMP and the (Sp)- and (Rp)-diastereoisomers of adenosine cyclic 3':5'-phosphorothioate by bovine heart and baker's-yeast cyclic AMP phosphodiesterases. *Biochem J* 203:461–470
- Jeon KI, Xu X, Aizawa T, Lim JH, Jono H, Kwon DS, Abe J, Berk BC, Li JD, Yan C (2010) Vinpocetine inhibits NF-kappaB-dependent inflammation via an IKK-dependent but PDE-independent mechanism. *Proc Natl Acad Sci USA* 107:9795–9800
- Jin SL, Swinnen JV, Conti M (1992) Characterization of the structure of a low Km, rolipram-sensitive cAMP phosphodiesterase. Mapping of the catalytic domain. *J Biol Chem* 267:18929–18939
- Jin SC, Richard FJ, Kuo WP, D'Ercole AJ, Conti M (1999) Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D- deficient mice. *Proc Natl Acad Sci USA* 96:11998–12003
- Juilfs DM, Fülle HJ, Zhao AZ, Houslay MD, Garbers DL, Beavo JA (1997) A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway. *Proc Natl Acad Sci USA* 94:3388–3395
- Kalgutkar AS, Choo E, Taylor TJ, Marfat A (2004) Disposition of CP-671, 305, a selective phosphodiesterase 4 inhibitor in preclinical species. *Xenobiotica* 34:755–770
- Kambayashi J, Liu Y, Sun B, Shakur Y, Yoshitake M, Czerwiec F (2003) Cilostazol as a unique antithrombotic agent. *Curr Pharm Des* 9:2289–2302

- Kamayashi J, Shakur Y, Liu Y (2006) Bench to bedside: multiple actions of the PDE3 inhibitor cilostazol. In: Beavo JA, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, pp 627–648
- Kang KK, Ahn GJ, Sohn YS, Ahn BO, Kim WB (2003) DA-8159, a new PDE5 inhibitor, attenuates the development of compensatory right ventricular hypertrophy in a rat model of pulmonary hypertension. *J Int Med Res* 31:517–528
- Kass DA, Champion HC, Beavo JA (2007) Phosphodiesterase type 5: expanding roles in cardiovascular regulation. *Circ Res* 101:1084–1095
- Ke H, Wang EH (2007a) Structure, catalytic mechanism, and inhibitor selectivity of cyclic nucleotide phosphodiesterases. In: Beavo JA, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. Taylor & Francis Group, LLC, Boca Raton, pp 607–626
- Ke H, Wang H (2007b) Crystal structures of phosphodiesterases and implications on substrate specificity and inhibitor selectivity. *Curr Top Med Chem* 7:391–403
- Keravis T, Lugnier C (2010) Cyclic nucleotide phosphodiesterases (PDE) and peptide motifs. *Curr Pharm Des* 16(9):1114–1125
- Kim D, Rybalkin SD, Pi X, Wang Y, Zhang C, Munzel T, Beavo JA, Berk BC, Yan C (2001) Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance. *Circulation* 104:2338–2343
- Klabunde RE (1983) Dipyridamole inhibition of adenosine metabolism in human blood. *Eur J Pharmacol* 93:21–26
- Kobayashi M, Nasuhara Y, Betsuyaku T, Shibuya E, Tanino Y, Tanino M, Takamura K, Nagai K, Hosokawa T, Nishimura M (2004) Effect of low-dose theophylline on airway inflammation in COPD. *Respirology* 9:249–254
- Labiberte F, Han Y, Govindarajan A, Giroux A, Liu S, Bobechko B, Lario P, Bartlett A, Gorseth E, Gresser M, Huang Z (2000) Conformational difference between PDE4 apoenzyme and holoenzyme. *Biochemistry* 39:6449–6458
- Labiberte F, Liu S, Gorseth E, Bobechko B, Bartlett A, Lario P, Gresser MJ, Huang Z (2002) In vitro PKA phosphorylation-mediated human PDE4A4 activation. *FEBS Lett* 512:205–208
- Li X, Baillie GS, Houslay MD (2009) Mdm2 directs the ubiquitination of β arrestin-sequestered cAMP phosphodiesterase-4D5. *J Biol Chem* 284:16170–16182
- Lim J, Pahlke G, Conti M (1999) Activation of the cAMP-specific phosphodiesterase PDE4D3 by phosphorylation. Identification and function of an inhibitory domain. *J Biol Chem* 274:19677–19685
- Liu S, Mansour MN, Dillman KS, Perez JR, Danley DE, Aeed PA, Simons SP, Lemotte PK, Menniti FS (2008) Structural basis for the catalytic mechanism of human phosphodiesterase 9. *Proc Natl Acad Sci USA* 105:13309–13314
- Livi GP, Kmetz P, McHale MM, Cieslinski LB, Sathe GM, Taylor DP, Davis RL, Torphy TJ, Balcarek JM (1990) Cloning and expression of cDNA for a human low-K_m, rolipram-sensitive cyclic AMP phosphodiesterase. *Mol Cell Biol* 10:2678–2686
- Lukowski R, Rybalkin SD, Loga F, Leiss V, Beavo JA, Hofmann F (2010) Cardiac hypertrophy is not amplified by deletion of cGMP-dependent protein kinase I in cardiomyocytes. *Proc Natl Acad Sci USA* 107:5646–5651
- Lynch MJ, Baillie GS, Mohamed A, Li X, Maisonneuve C, Klussmann E, van Heeke G, Houslay MD (2005) RNA Silencing Identifies PDE4D5 as the Functionally Relevant cAMP Phosphodiesterase Interacting with β Arrestin to Control the Protein Kinase A/AKAP79-mediated Switching of the β 2-Adrenergic Receptor to Activation of ERK in HEK293B2 Cells. *J Biol Chem* 280:33178–33189
- MacFarland RT, Zelus BD, Beavo JA (1991) High concentrations of a cGMP-stimulated phosphodiesterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. *J Biol Chem* 266:136–142
- Martinez SE, Beavo JA, Hol WG (2002a) GAF domains: two billion year old molecular switches that bind cyclic nucleotides. *Mol Interv* 2:317–323

- Martinez SE, Wu AY, Glavas NA, Tang XB, Turley S, Hol WG, Beavo JA (2002b) The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding. *Proc Natl Acad Sci USA* 99:13260–13265
- Martinez SE, Bruder S, Schultz A, Zheng N, Schultz JE, Beavo JA, Linder JU (2005) Crystal structure of the tandem GAF domains from a cyanobacterial adenylyl cyclase: modes of ligand binding and dimerization. *Proc Natl Acad Sci USA* 102:3082–3087
- Martinez SE, Heikaus CC, Klevit RE, Beavo JA (2008) The structure of the GAF A domain from phosphodiesterase 6C reveals determinants of cGMP binding, a conserved binding surface, and a large cGMP-dependent conformational change. *J Biol Chem* 283:25913–25919
- McCahill A, McSorley T, Huston E, Hill EV, Lynch MJ, Gall I, Keryer G, Lygren B, Tasken K, van Heeke G, Houslay MD (2005) In resting COS1 cells a dominant negative approach shows that specific, anchored PDE4 cAMP phosphodiesterase isoforms gate the activation, by basal cyclic AMP production, of AKAP-tethered protein kinase A type II located in the centrosomal region. *Cell Signal* 17:1158–1173
- McPhee I, Yarwood SJ, Huston E, Scotland G, Beard MB, Ross AH, Houslay ES, Houslay MD (1999) Association with the src family tyrosyl kinase LYN triggers a conformational change in the catalytic region of human cAMP-specific phosphodiesterase HSPDE4A4B: consequences for rolipram inhibition. *J Biol Chem* 274:11796–11810
- Mery PF, Pavoine C, Pecker F, Fischmeister R (1995) Erythro-9-(2-hydroxy-3-nonyl)adenine inhibits cyclic GMP-stimulated phosphodiesterase in isolated cardiac myocytes. *Mol Pharmacol* 48:121–130
- Miller CL, Oikawa M, Cai Y, Wojtovich AP, Nagel DJ, Xu X, Xu H, Florio V, Rybalkin SD, Beavo JA, Chen YF, Li JD, Blaxall BC, Abe J, Yan C (2009) Role of Ca²⁺/calmodulin-stimulated cyclic nucleotide phosphodiesterase 1 in mediating cardiomyocyte hypertrophy. *Circ Res* 105:956–964
- Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A, Huston E, Hannawacker A, Lohse MJ, Pozzan T, Houslay MD, Zaccolo M (2004) Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. *Circ Res* 95:67–75
- Mongillo M, Tocchetti CG, Terrin A, Lissandron V, Cheung Y-F, Dostmann WR, Pozzan T, Kass DA, Paolocci N, Houslay MD, Zaccolo M (2006) Compartmentalized phosphodiesterase-2 (PDE2) activity blunts β -adrenergic cardiac inotropy via a β_3 -adrenoceptor/NO/cGMP dependent pathway. *Circ Res* 98:226–234
- Mullershausen F, Russwurm M, Friebe A, Koesling D (2004) Inhibition of phosphodiesterase type 5 by the activator of nitric oxide-sensitive guanylyl cyclase BAY 41-2272. *Circulation* 109:1711–1713
- Murdoch H, Mackie S, Collins DM, Hill EV, Bolger GB, Klussmann E, Porteous DJ, Millar JK, Houslay MD (2007) Isoform-selective susceptibility of DISC1/phosphodiesterase-4 complexes to dissociation by elevated intracellular cAMP levels. *J Neurosci* 27:9513–9524
- Nagayama T, Zhang M, Hsu S, Takimoto E, Kass DA (2008) Sustained soluble guanylate cyclase stimulation offsets nitric-oxide synthase inhibition to restore acute cardiac modulation by sildenafil. *J Pharmacol Exp Ther* 326:380–387
- Obermolte R, Bhakta S, Alvarez R, Bach C, Zuppan P, Mulkins M, Jarnagin K, Shelton ER (1993) The cDNA of a human lymphocyte cyclic-AMP phosphodiesterase (PDE IV) reveals a multi-gene family. *Gene* 129:239–247
- Ohshiro H, Tonai-Kachi H, Ichikawa K (2008) GPR35 is a functional receptor in rat dorsal root ganglion neurons. *Biochem Biophys Res Commun* 365:344–348
- Omburo GA, Jacobitz S, Torphy TJ, Colman RW (1998) Critical role of conserved histidine pairs HNXXH and HDXXH in recombinant human phosphodiesterase 4A. *Cell Signal* 10:491–497
- Omori K, Kotera J (2006) PDE11. In: Beavo JA, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, pp 255–274
- Omori K, Kotera J (2007) Overview of PDEs and their regulation. *Circ Res* 100:309–327

- Owen DR, Walker JK, Jon Jacobsen E, Freskos JN, Hughes RO, Brown DL, Bell AS, Brown DG, Phillips C, Mischke BV, Molyneux JM, Fobian YM, Heasley SE, Moon JB, Stallings WC, Joseph Rogier D, Fox DN, Palmer MJ, Ringer T, Rodriguez-Lens M, Cabbage JW, Bleviss-Bal RM, Benson AG, Acker BA, Maddux TM, Tollefson MB, Bond BR, Macinnes A, Yu Y (2009) Identification, synthesis and SAR of amino substituted pyrido[3, 2b]pyrazinones as potent and selective PDE5 inhibitors. *Bioorg Med Chem Lett* 19:4088–4091
- Pandit J, Forman MD, Fennell KF, Dillman KS, Menniti FS (2009) Mechanism for the allosteric regulation of phosphodiesterase 2A deduced from the X-ray structure of a near full-length construct. *Proc Natl Acad Sci USA* 106:18225–18230
- Penmatsa H, Zhang W, Yarlagadda S, Li C, Conoley VG, Yue J, Bahouth SW, Buddington RK, Zhang G, Nelson DJ, Sonecha MD, Manganiello V, Wine JJ, Naren AP (2010) Compartmentalized cyclic adenosine 3', 5'-monophosphate at the plasma membrane clusters PDE3A and cystic fibrosis transmembrane conductance regulator into microdomains. *Mol Biol Cell* 21:1097–1110
- Poppe H, Rybalkin SD, Rehmann H, Hinds TR, Tang XB, Christensen AE, Schwede F, Genieser HG, Bos JL, Doskeland SO, Beavo JA, Butt E (2008) Cyclic nucleotide analogs as probes of signaling pathways. *Nat Methods* 5:277–278
- Puxeddu E, Uhart M, Li CC, Ahmad F, Pacheco-Rodriguez G, Manganiello VC, Moss J, Vaughan M (2009) Interaction of phosphodiesterase 3A with brefeldin A-inhibited guanine nucleotide-exchange proteins BIG1 and BIG2 and effect on ARF1 activity. *Proc Natl Acad Sci USA* 106:6158–6163
- Rabe KF, Magnussen H, Dent G (1995) Theophylline and selective PDE inhibitors as bronchodilators and smooth muscle relaxants. *Eur Respir J* 8:637–642
- Rich TC, Fagan KA, Tse TE, Schaack J, Cooper DM, Karpen JW (2001a) A uniform extracellular stimulus triggers distinct cAMP signals in different compartments of a simple cell. *Proc Natl Acad Sci USA* 98:13049–13054
- Rich TC, Tse TE, Rohan JG, Schaack J, Karpen JW (2001b) In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. *J Gen Physiol* 118:63–78
- Rich TC, Xin W, Mehats C, Hassell KA, Piggott L, Le X, Karpen JW, Conti M (2006) Cellular mechanisms underlying prostaglandin-induced transient cAMP signals near the plasma membrane of HEK-293 cells. *Am J Physiol Cell Physiol* 292:C319–C331
- Richter W, Conti M (2002) Dimerization of the type 4 cAMP-specific phosphodiesterases is mediated by the upstream conserved regions (UCRs). *J Biol Chem* 277:40212–40221
- Richter W, Conti M (2004) The oligomerization state determines regulatory properties and inhibitor sensitivity of type 4 cAMP-specific phosphodiesterases. *J Biol Chem* 279:30338–30348
- Rocque WJ, Holmes WD, Patel IR, Dougherty RW, Ittoop O, Overton L, Hoffman CR, Wisely GB, Willard DH, Luther MA (1997) Detailed characterization of a purified type 4 phosphodiesterase, HSPDE4B2B: differentiation of high- and low-affinity (R)-rolipram binding. *Protein Exp Purif* 9:191–202
- Rybalkin SD, Bornfeldt KE, Sonnenburg WK, Rybalkina IG, Kwak KS, Hanson K, Krebs EG, Beavo JA (1997) Calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1C) is induced in human arterial smooth muscle cells of the synthetic, proliferative phenotype. *J Clin Invest* 100:2611–2621
- Rybalkin SD, Yan C, Bornfeldt KE, Beavo JA (2003) Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ Res* 93:280–291
- Rybalkina IG, Tang XB, Rybalkin SD (2010) Multiple affinity states of cGMP-specific phosphodiesterase for sildenafil inhibition defined by cGMP-dependent and cGMP-independent mechanisms. *Mol Pharmacol* 77:670–677
- Salanova M, Jin SC, Conti M (1998) Heterologous expression and purification of recombinant rolipram-sensitive cyclic AMP-specific phosphodiesterases. *Methods* 14:55–64
- Saldou N, Baecker PA, Li B, Yuan Z, Obermolte R, Ratzliff J, Osen E, Jarnagin K, Shelton ER (1998) Purification and physical characterization of cloned human cAMP phosphodiesterases PDE-4D and -4C. *Cell Biochem Biophys* 28:187–217

- Scapin G, Patel SB, Chung C, Varnerin JP, Edmondson SD, Mastracchio A, Parmee ER, Singh SB, Becker JW, Van der Ploeg LH, Tota MR (2004) Crystal structure of human phosphodiesterase 3B: atomic basis for substrate and inhibitor specificity. *Biochemistry* 43:6091–6100
- Schaper W (2005) Dipyridamole, an underestimated vascular protective drug. *Cardiovasc Drugs Ther* 19:357–363
- Schultz JE, Dunkern T, Gawlitta-Gorka E, Sorg G (2011) The GAF-tandem domain of phosphodiesterase 5 as a potential drug target. Francis SH, Conti M, Houslay MD (eds) *Phosphodiesterases as drug targets*. Springer, Heidelberg
- Scotland G, Houslay MD (1995) Chimeric constructs show that the unique N-terminal domain of the cyclic AMP phosphodiesterase RD1 (rPDE-IV_{A1};RNPDE4A1A) can confer membrane association upon the normally cytosolic protein chloramphenicol acetyl transferase (CAT). *Biochem J* 308:673–681
- Sehkar KR, Grondin P, Francis SH, Corbin JD (1996) Design and synthesis of xanthines and cyclic GMP analogues as potent inhibitors of PDE5. In: Schudt C, Dent G, Rabe KF (eds) *Phosphodiesterase inhibitors*. Academic, New York, pp 135–146
- Serebruany V, Sabaeva E, Booze C, Atar OD, Eisert C, Hanley D (2009) Distribution of dipyridamole in blood components among post-stroke patients treated with extended release formulation. *Thromb Haemost* 102:538–543
- Sette C, Conti M (1996) Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation. *J Biol Chem* 271:16526–16534
- Shakur Y, Pryde J, Houslay MD (1993) Engineered deletion of the unique N-terminal domain of the cyclic AMP specific phosphodiesterase RD1 prevents plasma membrane association and the attainment of enhanced thermostability without altering its sensitivity to inhibition by rolipram. *Biochem J* 292:677–686
- Smith KJ, Scotland G, Beattie J, Trayer IP, Houslay MD (1996) Determination of the structure of the N-terminal splice region of the cyclic AMP-specific phosphodiesterase RD1 (RNPDE4A1) by ¹H-NMR and identification of the membrane association domain using chimeric constructs. *J Biol Chem* 271:16703–16711
- Smith KJ, Baillie GS, Hyde EI, Li X, Houslay TM, McCahill A, Dunlop AJ, Bolger GB, Klusmann E, Adams DR, Houslay MD (2007) ¹H NMR structural and functional characterisation of a cAMP-specific phosphodiesterase-4D5 (PDE4D5) N-terminal region peptide that disrupts PDE4D5 interaction with the signalling scaffold proteins, beta-arrestin and RACK1. *Cell Signal* 19:2612–2624
- Souness JE, Rao S (1997) Proposal for pharmacologically distinct conformers of PDE4 cyclic AMP phosphodiesterases. *Cell Signal* 9:227–236
- Stasch JP, Hobbs AJ (2009) NO-independent, haem-dependent soluble guanylate cyclase stimulators. *Handb Exp Pharmacol* 191:277–308
- Steinberg SF, Brunton LL (2001) Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annu Rev Pharmacol Toxicol* 41:751–773
- Sudo T, Tachibana K, Toga K, Tochizawa S, Inoue Y, Kimura Y, Hidaka H (2000) Potent effects of novel anti-platelet aggregatory cilostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity. *Biochem Pharmacol* 59:347–356
- Sullivan M, Egerton M, Shakur Y, Marquardsen A, Houslay MD (1994) Molecular cloning and expression, in both COS-1 cells and *S. cerevisiae*, of a human cytosolic type-IVA, cyclic AMP specific phosphodiesterase (hPDE-IVA-h6.1). *Cell Signal* 6:793–812
- Sung BJ, Hwang KY, Jeon YH, Lee JI, Heo YS, Kim JH, Moon J, Yoon JM, Hyun YL, Kim E, Eum SJ, Park SY, Lee JO, Lee TG, Ro S, Cho JM (2003) Structure of the catalytic domain of human phosphodiesterase 5 with bound drug molecules. *Nature* 425:98–102
- Takimoto E, Champion HC, Belardi D, Moslehi J, Mongillo M, Mergia E, Montrose DC, Isoda T, Auferio K, Zaccolo M, Dostmann WR, Smith CJ, Kass DA (2005a) cGMP catabolism by phosphodiesterase 5A regulates cardiac adrenergic stimulation by NOS3-dependent mechanism. *Circ Res* 96:100–109

- Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, Bedja D, Gabrielson KL, Wang Y, Kass DA (2005b) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* 11:214–222
- Taniguchi Y, Tonai-Kachi H, Shinjo K (2006) Zaprinast, a well-known cyclic guanosine monophosphate-specific phosphodiesterase inhibitor, is an agonist for GPR35. *FEBS Lett* 580:5003–5008
- Tasken KA, Collas P, Kemmner WA, Witczak O, Conti M, Tasken K (2001) Phosphodiesterase 4D and protein kinase a type II constitute a signaling unit in the centrosomal area. *J Biol Chem* 276:21999–22002
- Tenor H, Hatzelmann A, Beume R, Lahu G, Zech K, Bethke TD (2011) Pharmacology, clinical efficacy and tolerability of PDE4 inhibitors: Impact of human pharmacokinetics. Francis SH, Conti M, Houslay MD (eds) *Phosphodiesterases as drug targets*. Springer, Heidelberg
- Thomas MK, Francis SH, Beebe SJ, Gettys TW, Corbin JD (1992) Partial mapping of cyclic nucleotide sites and studies of regulatory mechanisms of phosphodiesterases using cyclic nucleotide analogues. *Adv Second Messenger Phosphoprotein Res* 25:45–53
- Thompson WJ (1991) Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function. *Pharmacol Ther* 51:13–33
- Tian G, Rocque WJ, Wiseman JS, Thompson IZ, Holmes WD, Domanico PL, Stafford JA, Feldman PL, Luther MA (1998) Dual inhibition of human type 4 phosphodiesterase isostates by (R, R)-(+/-)-methyl 3-acetyl-4-[3-(cyclopentyloxy)-4-methoxyphenyl]-3-methyl-1-pyrrolidinecarboxylate. *Biochemistry* 37:6894–6904
- Toque HA, Teixeira CE, Priviero FB, Morganti RP, Antunes E, De Nucci G (2008) Vardenafil, but not sildenafil or tadalafil, has calcium-channel blocking activity in rabbit isolated pulmonary artery and human washed platelets. *Br J Pharmacol* 154:787–796
- Torphy TJ (1998) Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. *Am J Respir Crit Care Med* 157:351–370
- Trifileff A, Wyss D, Walker C, Mazzoni L, Hersperger R (2002) Pharmacological profile of a novel phosphodiesterase 4 inhibitor, 4-(8-benzo[1,2,5]oxadiazol-5-yl-[1,7]naphthyridin-6-yl)-benzoic acid (NVP-ABE171), a 1,7-naphthyridine derivative, with anti-inflammatory activities. *J Pharmacol Exp Ther* 301:241–248
- Turko IV, Ballard SA, Francis SH, Corbin JD (1999) Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. *Mol Pharmacol* 56:124–130
- Uhler MD (1993) Cloning and expression of a novel cyclic GMP-dependent protein kinase from mouse brain. *J Biol Chem* 268:13586–13591
- Vandeput F, Krall J, Ockaili R, Salloum FN, Florio V, Corbin JD, Francis SH, Kukreja RC, Movsesian MA (2009) cGMP-hydrolytic activity and its inhibition by sildenafil in normal and failing human and mouse myocardium. *J Pharmacol Exp Ther* 330:884–891
- Verhoest PR, Chapin DS, Corman M, Fonseca K, Harms JF, Hou X, Marr ES, Menniti FS, Nelson F, O'Connor R, Pandit J, Proulx-Lafrance C, Schmidt AW, Schmidt CJ, Suiciak JA, Liras S (2009) Discovery of a Novel Class of Phosphodiesterase 10A Inhibitors and Identification of Clinical Candidate 2-[4-(1-Methyl-4-pyridin-4-yl-1H-pyrazol-3-yl)-phenoxy-methyl]-quinoline (PF-2545920) for the Treatment of Schizophrenia (dagger) dagger Coordinates of the PDE10A crystal structures have been deposited in the Protein Data Bank for compound 1 (3HQW), 2 (3HQY), 3 (3HQW) and 9 (3HR1). *J Med Chem* 52:5188
- Walseth TF, Gander JE, Eide SJ, Krick TP, Goldberg ND (1983) 18O labeling of adenine nucleotide alpha-phosphoryls in platelets. Contribution by phosphodiesterase-catalyzed hydrolysis of cAMP. *J Biol Chem* 258:1544–1558
- Wang P, Myers JG, Wu P, Cheewatrakoolpong B, Egan RW, Billah MM (1997) Expression, purification, and characterization of human cAMP-specific phosphodiesterase (PDE4) subtypes A, B, C, and D. *Biochem Biophys Res Commun* 234:320–324
- Wang H, Liu Y, Chen Y, Robinson H, Ke H (2005) Multiple elements jointly determine inhibitor selectivity of cyclic nucleotide phosphodiesterases 4 and 7. *J Biol Chem* 280:30949–30955

- Wang H, Liu Y, Hou J, Zheng M, Robinson H, Ke H (2007) Structural insight into substrate specificity of phosphodiesterase 10. *Proc Natl Acad Sci USA* 3;104:5782–5787
- Wang H, Liu Y, Huai Q, Cai J, Zoraghi R, Francis SH, Corbin JD, Robinson H, Xin Z, Lin G, Ke H (2006) Multiple conformations of phosphodiesterase-5: implications for enzyme function and drug development. *J Biol Chem* 281:21469–21479
- Wang H, Peng MS, Chen Y, Geng J, Robinson H, Houslay MD, Cai J, Ke H (2007) Structures of the four subfamilies of phosphodiesterase-4 provide insight into the selectivity of their inhibitors. *Biochem J* 408:193–201
- Wang H, Yan Z, Yang S, Cai J, Robinson H, Ke H (2008) Kinetic and structural studies of phosphodiesterase-8A and implication on the inhibitor selectivity. *Biochemistry* 47:12760–12768
- Wang H, Ye M, Robinson H, Francis SH, Ke H (2008) Conformational variations of both phosphodiesterase-5 and inhibitors provide the structural basis for the physiological effects of vardenafil and sildenafil. *Mol Pharmacol* 73:104–110
- Weeks JL 2nd, Zoraghi R, Francis SH, Corbin JD (2007) N-Terminal domain of phosphodiesterase-11A4 (PDE11A4) decreases affinity of the catalytic site for substrates and tadalafil, and is involved in oligomerization. *Biochemistry* 46:10353–10364
- Wernet W, Flockerzi V, Hofmann F (1989) The cDNA of the two isoforms of bovine cGMP-dependent protein kinase. *FEBS Lett* 251:191–196
- Wilson LS, Elbatamy HS, Crawley SW, Bennett BM, Maurice DH (2008) Compartmentation and compartment-specific regulation of PDE5 by protein kinase G allows selective cGMP-mediated regulation of platelet functions. *Proc Natl Acad Sci USA* 105:13650–13655
- Wu AY, Tang XB, Martinez SE, Ikeda K, Beavo JA (2004) Molecular determinants for cyclic nucleotide binding to the regulatory domains of phosphodiesterase 2A. *J Biol Chem* 279:37928–37938
- Xu RX, Hassell AM, Vanderwall D, Lambert MH, Holmes WD, Luther MA, Rocque WJ, Milburn MV, Zhao Y, Ke H, Nolte RT (2000) Atomic structure of PDE4: insights into phosphodiesterase mechanism and specificity. *Science* 288:1822–1825
- Xu RX, Rocque WJ, Lambert MH, Vanderwall DE, Luther MA, Nolte RT (2004) Crystal structures of the catalytic domain of phosphodiesterase 4B complexed with AMP, 8-Br-AMP, and rolipram. *J Mol Biol* 337:355–365
- Yamamoto T, Yamamoto S, Osborne JC Jr, Manganiello VC, Vaughan M, Hidaka H (1983) Complex effects of inhibitors on cyclic GMP-stimulated cyclic nucleotide phosphodiesterase. *J Biol Chem* 258:14173–14177
- Yan C, Zhao AZ, Bentley JK, Beavo JA (1996) The calmodulin-dependent phosphodiesterase gene PDE1C encodes several functionally different splice variants in a tissue-specific manner. *J Biol Chem* 271:25699–25706
- Yarwood SJ, Steele MR, Scotland G, Houslay MD, Bolger GB (1999) The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. *J Biol Chem* 274:14909–14917
- Zaccolo M, Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* 295:1711–1715
- Zhang KYJ (2006) Crystal structure of phosphodiesterase families and the potential for rational drug design. In: Beavo J, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterase in health and disease*. CRC Press, Boca Raton, pp 583–605
- Zhang W, Ke H, Colman RW (2002) Identification of interaction sites of cyclic nucleotide phosphodiesterase type 3A with milrinone and cilostazol using molecular modeling and site-directed mutagenesis. *Mol Pharmacol* 62:514–520
- Zhang X, Feng Q, Cote RH (2005) Efficacy and selectivity of phosphodiesterase-targeted drugs in inhibiting photoreceptor phosphodiesterase (PDE6) in retinal photoreceptors. *Invest Ophthalmol Vis Sci* 46:3060–3066
- Zhang XJ, Skiba NP, Cote RH (2009) Structural requirements of the photoreceptor phosphodiesterase $\{\gamma\}$ -subunit for inhibition of rod PDE6 holoenzyme and for its activation by transducin. *J Biol Chem* 285:4455–4463

- Zhao Y, Zhang HT, O'Donnell JM (2003) Inhibitor binding to type 4 phosphodiesterase (PDE4) assessed using [3H]piclamilast and [3H]rolipram. *J Pharmacol Exp Ther* 305:565–572
- Zhu B, Strada SJ (2007) The novel functions of cGMP-specific phosphodiesterase 5 and its inhibitors in carcinoma cells and pulmonary/cardiovascular vessels. *Curr Top Med Chem* 7: 437–454
- Zoraghi R, Corbin JD, Francis SH (2004) Properties and functions of GAF domains in cyclic nucleotide phosphodiesterases and other proteins. *Mol Pharmacol* 65:267–278
- Zoraghi R, Bessay EP, Corbin JD, Francis SH (2005) Structural and functional features in human PDE5A1 regulatory domain that provide for allosteric cGMP binding, dimerization, and regulation. *J Biol Chem* 280:12051–12063
- Zoraghi R, Francis SH, Corbin JD (2007) Critical amino acids in phosphodiesterase-5 catalytic site that provide for high-affinity interaction with cGMP and inhibitors. *Biochemistry* 46: 13554–13563

Pharmacology, Clinical Efficacy, and Tolerability of Phosphodiesterase-4 Inhibitors: Impact of Human Pharmacokinetics

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Karl Zech, and Thomas D. Bethke

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Abstract Since more than two decades anti-inflammatory effects of inhibitors of phosphodiesterase-4 have been described in numerous cellular and animal studies and were finally confirmed in clinical trials. The path from an early, pioneering study with Ro20-1724 showing reduction of psoriatic plaque size in 1979 to modern PDE4 inhibitors such as oral apremilast in development for psoriasis, the

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inhaled PDE4 inhibitor GSK256066 in development for asthma and COPD and finally roflumilast, the first PDE4 inhibitor approved and currently marketed as an oral, once-daily remedy for severe COPD was marked by large progress in chemical optimization based on improved understanding of PDE4 biology and drug-like properties determining the appropriate pharmacokinetic profile. In this chapter aspects of the pharmacology and clinical efficacy of PDE4 inhibitors, which have been in clinical development over the years are summarized with specific emphasis on their clinical pharmacokinetic properties.

Keywords Apremilast · GSK256066 · PDE4 Inhibitors · Pharmacokinetics · Roflumilast

1 Introduction

Phosphodiesterase 4 (PDE4) is abundantly expressed in a broad array of cells governing inflammatory responses and structural remodeling. Over the last two decades, an ever-increasing amount of evidence from cellular and animal studies indicated potential benefits from selective PDE4 inhibitors in numerous ailments. Among them were respiratory disorders such as chronic obstructive pulmonary disease (COPD), asthma, or idiopathic pulmonary fibrosis. The major clinical breakthrough with PDE4 inhibitors was in COPD, a chronic respiratory disorder mostly developing over decades in about 20–30% of tobacco smokers, regardless of later smoking cessation. COPD is characterized by a chronic, progressive loss in lung function associated with sudden acute exacerbations. Major disease mechanisms in COPD involve a characteristic pulmonary inflammatory response, structural remodeling encompassing lung parenchymal destruction (emphysema), and small airway peribronchial fibrosis, as well as malfunction of the mucociliary apparatus (Barnes and Rennard 2008; Hogg and Timens 2009).

Interestingly, in large clinical trials the once-daily (500 µg per day), oral PDE4 inhibitor Roflumilast significantly improved lung function and reduced the rate of acute exacerbations in patients with severe COPD (Calverley et al. 2009), especially when added to long-acting bronchodilators such as salmeterol or tiotropium (Fabbri et al. 2009). In contrast, the clinical effects of oral PDE4 inhibitors are not associated with acute bronchodilation (Harbinson et al. 1997; Engelstaetter et al. 2002; Grootendorst et al. 2003). On the basis of its clinical effectiveness in COPD, roflumilast has recently received market authorization by the European Medicinal's Agency as the first-in-class PDE4 inhibitor for the maintenance treatment of severe COPD associated with chronic bronchitis in adult patients with a history of frequent exacerbations as an add-on to bronchodilator treatment.

Besides affecting the lungs, there is increasing awareness of extrapulmonary components of COPD, reflected as systemic inflammation and comorbidities (Agusti and Soriano 2008; Barnes and Celli 2009). The idea may be proposed that oral PDE4 inhibitors by virtue of their anti-inflammatory potential and their ability

to curb hyperactive structural cells may potentially alleviate these extrapulmonary manifestations. This hypothesis should be challenged in future investigations.

The history of PDE4 inhibitors dates back at least three decades and perhaps the first clinical proof-of-concept study was with Ro 20-1724 that was later characterized as a PDE4 inhibitor. This compound reduced plaque size in psoriasis patients when administered topically as reported by Stawiski and colleagues in 1979 (Stawiski et al. 1979). Immediately following the discovery of PDE4 in the 1980s, there was an exponential interest in PDE4 inhibitors. This was particularly attributed to the excellent drugability of PDE4 as a target and the rapidly accumulating evidence on the potential of PDE4 inhibitors to mitigate inflammation. However, first-generation PDE4 inhibitors comprising Ro 20-1724, rolipram, or denbufylline were associated with side effects such as nausea and vomiting that prevented their clinical development. More recent developments, including cilomilast, roflumilast, and apremilast (Fig. 1), were specifically designed to temper this adverse profile and have been investigated in large clinical trials, mostly in COPD or asthma, or with apremilast in psoriasis.

The wealth of knowledge on the biology of PDE4, medicinal chemistry of selective PDE4 inhibitors, and the abundance of cellular and animal effects associated with PDE4 inhibitors have been exhaustively summarized and discussed in excellent and authoritative recent reviews (Bender and Beavo 2006; Conti and Beavo 2007; Boswell-Smith and Spina 2007; Spina 2008; Pagès et al. 2009; Press and Banner 2009). This chapter, however, is an attempt to collate our current knowledge and opinion on the clinical pharmacokinetics of PDE4 inhibitors and to discuss, where possible, how pharmacokinetic profiles might modify clinical

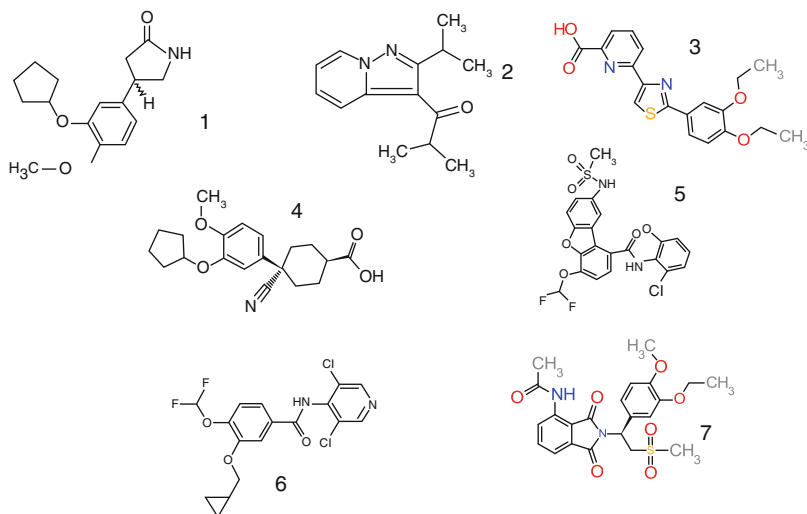


Fig. 1 Chemical structures of selected oral PDE4 inhibitors, which have been in clinical development. (1) rolipram; (2) ibudilast; (3) tetomilast; (4) cilomilast; (5) oglemilast, (6) roflumilast, (7) apremilast

efficacy. For the PDE4 inhibitors discussed (rolipram (**1**), ibudilast (**2**), tetomilast (**3**), cilomilast (**4**), oglemilast (**5**), roflumilast (**6**), and apremilast (**7**); Fig. 1), a very brief summary of cellular, animal, and clinical findings will be added.

2 Pharmacology and Clinical Experience

2.1 Rolipram

The arylpyrrolidinone rolipram (**1**) was among the first discovered set of highly selective PDE4 inhibitors. This compound was investigated in clinical development for the treatment of depression (Zeller et al. 1984; Laux et al. 1988; Fleischhacker et al. 1992). Rolipram readily crosses the blood–brain barrier and has been found to improve neurotransmission in central noradrenergic neurons. This observation may account for the compound's antidepressive effects. Although modest antidepressive efficacy for rolipram was reported in clinical settings, the extent of nausea and vomiting caused further development to be abandoned. As the archetype PDE4 inhibitor with a rather acceptable pharmacokinetic profile in rats or mice following i.p. or p.o. administration, rolipram excelled as a tool compound for characterizing the putative potential of PDE4 inhibitors in countless disease-relevant cellular and animal models but also to sharpen insights into the nature of PDE4-related adverse effects such as nausea and vomiting.

On the basis of promising results to alleviate symptoms in experimental autoimmune encephalomyelitis (EAE) rodent models, the effects of rolipram were explored in a very small number of patients afflicted with multiple sclerosis. Although rolipram (maximal dose of 9 mg per day) exerted inhibitory effects on peripheral blood mononuclear cells, the primary outcome measure (contrast-enhanced lesions in magnetic resonance imaging (MRI) reflecting blood–brain barrier disruption secondary to brain inflammatory activity) worsened rather than improved. Along with poor tolerability, this finding accounted for premature termination of that study (Bielekova et al. 2009). The reasons for the enhanced brain inflammatory activity with rolipram are not entirely clear; however, one may consider that not only TH1 cells but also other T-cell subpopulations such as TH17 cells are now considered being critical orchestrators in multiple sclerosis. Authors indicated the study does not exclude that PDE4 inhibitors may exert neuroprotective or immunomodulatory effects independently of blood–brain barrier disruption. In any case, caution should be taken in investigations with PDE4 inhibitors in multiple sclerosis.

2.2 Ibudilast

Ibudilast, a pyrazolopyridine (**2**, Kyorin Pharmaceuticals), may be at best considered as a weak, mixed type PDE4, PDE10, and PDE3 inhibitor with IC_{50} in the low micromolar range and little specificity for PDE4 (Gibson et al. 2006; Huang et al.

2006). Ibudilast is registered in Japan for the treatment of asthma and poststroke dizziness (Ketas[®] and Pinatos[®], delayed release capsules) as well as for the treatment of ocular allergies with 0.01% ibudilast ophthalmic solution. The approved oral dose is 10 mg administered twice (for asthma) or three times daily (for the cerebrovascular condition).

Bronchodilator, vasodilator, antithrombotic, and anti-inflammatory effects are attributed to the drug. The rationale for the use of ibudilast in poststroke dizziness appears to be based on the assumption that this condition results from ongoing ischemia and that the vasodilating activity of ibudilast may improve this condition (Rolan et al. 2009).

In vitro effects of ibudilast include attenuation of leukotriene release (Ohtsu et al. 1989), diminished TNF- α or interferon (IFN)- γ production from T-cells (Feng et al. 2004), and decreased histamine release from mast cells (Choi et al. 1989). Recently, it was revealed that ibudilast is an allosteric inhibitor of macrophage migration inhibitory factor (MIF), besides inhibiting PDEs (Cho et al. 2010).

2.3 *Tetomilast*

Tetomilast (OPC-6535; Otsuka) (**3**) is a comparably weak PDE4 inhibitor (IC_{50} of 380 nM) with only about tenfold selectivity toward PDE3, which has been in clinical development for ulcerative colitis. In a phase II study in mild-to-moderate, active ulcerative colitis, the primary end point (reduction in “disease activity index”, a composite score, by at least three points within 8 weeks of treatment), was not achieved with a once-daily, oral dose of 25 or 50 mg per day (Keshavarzian et al. 2007). However, post hoc analyses in the subgroup with moderate activity revealed some symptomatic benefit. Tetomilast was advocated as being well tolerated by most individuals; however, 29% of patients assigned to the higher dose group experienced nausea and 6.5% vomiting, although these adverse events were mostly transient over the first weeks (Keshavarzian et al. 2007). In two more recent phase III trials (FACT 1 and FACT 2) in patients with moderately severe active ulcerative colitis, again the primary end point (a composite symptom score indicative of clinically meaningful improvement) was failed. Nausea was reported as the most common, dose-dependent adverse event (Keshavarzian et al. 2007). According to the company’s website, tetomilast remains under investigations in clinical studies for Crohn’s disease, but more recently, a clinical study in COPD has also been registered.

2.4 *Cilomilast*

Cilomilast (Ariflo, SB 207499, GSK) (**4**) is a second-generation, selective PDE4 inhibitor originally developed for the treatment of asthma, later for COPD, that was finally abandoned by GSK.

Cilomilast is more potent at inhibiting PDE4D (IC_{50} of 20 nM) compared to PDE4B (IC_{50} of 140 nM) (Giembycz 2001).

In this context, one may consider that in mice that were deficient for PDE4D, α_2 -adrenoceptor-induced anesthesia was shortened, which is conceived as a correlate to emesis in these non-emetic rodents (Robichaud et al. 2002). It was striking then that, contrary to expectations, the recently discovered highly PDE4D-selective, allosteric PDE4 inhibitors (Burgin et al. 2010) have almost no emetic potential in several animal species and did not show reduced duration of α_2 -adrenoceptor-induced anesthesia at doses associated with desired pharmacological effects (cognition enhancement) (Burgin et al. 2010). These findings were in contrast to rolipram that, however, suppresses catalytic activity of the different PDE4 subtypes with comparable potency.

In any case, PDE4D inhibition adds to the overall anti-inflammatory effects of a “balanced” PDE4 inhibitor, as this was exemplified in T-lymphocytes (Peter et al. 2007) or neutrophils (Ariga et al. 2004). “Balanced” PDE4 inhibitors are defined as those suppressing all PDE4 subtypes and splice variants with comparable potency.

As the cyclohexanecarboxylic acid substituent of cilomilast is deprotonated to the corresponding carboxylate anion at physiologic pH , drug penetration to the brain should be limited and the risk of CNS-related adverse events may be reduced.

Cilomilast showed anti-inflammatory effects in patients afflicted with COPD. In a 3-month trial in which patients received cilomilast at 15 mg BID versus placebo, the PDE4 inhibitor substantially reduced CD8+ T-cells and CD68+ macrophages in bronchial biopsies (Gamble et al. 2003). On the other hand, sputum neutrophil counts remained unaffected. No consistent anti-inflammatory effects could be determined in two additional studies of comparable design (Rennard et al. 2008).

In the phase III program involving patients with COPD treated with cilomilast (15 mg BID), improvements in FEV₁ ranged from 24 to 44 ml, but apparently this was not accompanied by consistent improvements in other clinical parameters. In one study, the PDE4 inhibitor significantly reduced functional residual capacity (FRC), a measure of hyperinflation by 290 ml; however, in another study this improvement (120 ml) remained below the level of significance (Rennard et al. 2008). Finally, the rate of acute exacerbations was reduced in two 24-week trials, but this was not confirmed in a later 1-year study that was designed to detect a reduction in exacerbations (Rennard et al. 2008).

2.5 Oglemilast

Oglemilast (GRC-3886, Glenmark) (**5**) is another potent (IC₅₀ PDE4B 0.3 nM, nonselective among PDE4 subtypes) and selective PDE4 inhibitor that has been undergoing clinical evaluation for COPD as an oral, once-daily remedy. While oglemilast was reported being non-emetic in ferrets (100 mg kg⁻¹) or dogs (12 mg kg⁻¹) at doses resulting in plasma levels (Vakkalanka et al. 2005) corresponding to almost maximum suppression of cellular effects related to PDE4 inhibition (LPS-induced TNF- α release in human whole blood and fMLP-induced superoxide formation in human PMN in the presence of 80% plasma), the compound failed

to reach primary end points in asthma and COPD trials (200–800 µg OD, p.o.), and, consequently, further development was terminated.

2.6 Roflumilast

Roflumilast (**6**, Nycomed) was developed as a novel, once-daily oral treatment for severe COPD that targets the underlying inflammatory disease processes (Hatzelmann and Schudt 2001; Bundschuh et al. 2001; Hatzelmann et al. 2010).

Roflumilast inhibits the PDE4 activity in human neutrophils (IC_{50} at 0.7 nM) without affecting any other isoenzyme of the 11 PDE families, even at 10,000-fold higher concentrations, and has a considerably higher potency for PDE4 inhibition than cilomilast (Hatzelmann and Schudt 2001; Hatzelmann et al. 2010).

Roflumilast N-oxide is the active metabolite of roflumilast sharing highly potent (IC_{50} at PDE4 of human neutrophils of 2 nM) and selective PDE4 inhibition with the parent compound (Hatzelmann and Schudt 2001).

Roflumilast and roflumilast N-oxide are “balanced” PDE4 inhibitors. In *in vitro* investigations, roflumilast and roflumilast N-oxide demonstrated a broad potential to reverse malfunctions of human inflammatory and structural cells related to COPD (such as neutrophils, macrophages, T-cells, airway epithelial cells, lung fibroblasts, bronchial and pulmonary artery smooth muscle cells, and endothelial cells). In mice studies, roflumilast alleviated the inflammatory response as well as lung parenchymal destruction following exposure to tobacco smoke over several months. Roflumilast further reduced the lung fibrotic response secondary to bleomycin in mice and rats. Finally, roflumilast mitigated pulmonary vascular remodeling and pulmonary hypertension in rats following chronic hypoxia or monocrotaline (see Hatzelmann et al. 2010 for review). The anti-inflammatory potential of roflumilast was confirmed in clinical studies. One study administered roflumilast at the standard dose of 500 µg per day over 4 weeks to human volunteers that were then exposed to lipopolysaccharide (LPS) by bronchoscopy-assisted segmental bronchial challenge (Hohlfeld et al. 2008). The resulting increase of neutrophils in the segmental lavage was reduced with roflumilast versus placebo by 39% (Hohlfeld et al. 2008). In another study recruiting patients afflicted with mild-to-moderate COPD, Grootendorst and colleagues (2007) found a reduction by approximately 35% in the number of neutrophils collected in induced sputum following a 4-week course of oral roflumilast, once daily at 500 µg per day. These anti-inflammatory effects may translate into the observed clinical efficacy in COPD. In four large clinical trials, roflumilast was shown to robustly (1) increase FEV1 by about 40–80 ml over 1 year, (2) reduce the rate of acute exacerbations in COPD by 15–21% (1 year), and (3) maintain its favorable effects on lung function and exacerbation rate when added to bronchodilators such as tiotropium or salmeterol (Calverley et al. 2009; Fabbri et al. 2009; Hanania et al. 2010). Retrospective analyses revealed that roflumilast can also reduce exacerbations and improve lung function when given concomitantly with inhaled corticosteroids in patients with severe COPD (Calverley et al. 2010).

2.7 Other Substances

2.7.1 Theophylline

The xanthine theophylline has been in use as a remedy for respiratory disorders for many decades. It was already known in 1957 that theophylline inhibits PDE activities, along with the discovery of cAMP by Earl Sutherland and Ted Rall. While the xanthine (together with caffeine as one pharmacologically active ingredient in coffee, described by Dr Hyde Salter in 1859 to alleviate asthma) was the first PDE inhibitor described, from our current perspective in 2010, it is a very weak (IC_{50} at PDE4 of 660 μ M) and unselective PDE inhibitor. Indeed, perhaps the only PDE component that may add to therapeutic effects of theophylline is inhibition of PDE3 (IC_{50} of 110 μ M) that accounts in part for bronchodilation.

At therapeutic plasma levels theophylline primarily diminishes the activity of phosphatidylinositol 3-kinase (PI3K)- δ following its cellular activation by reactive oxygen species (ROS), thus maintaining the activity of histone deacetylase-2 (HDAC2), which then supports to restore anti-inflammatory effectiveness of glucocorticoids (To et al. 2010). Steroid effectiveness is impaired in COPD probably as the result of the burden of ROS that prevails in this ailment (Barnes 2009). While ample affirmative evidence for this concept has been accumulated in cellular and animals studies, clinical investigations with the objective of defining whether theophylline, for example, reverses the resistance of sputum neutrophil accumulation to glucocorticoids in COPD are not yet conclusive (Cosio et al. 2009; Ford et al. 2010). At least in vitro, in alveolar macrophages from patients with COPD, theophylline at only 1 μ M (a concentration at which none of the PDEs is inhibited) that is ineffective alone was shown to partly restore the impaired inhibition of LPS-induced interleukin-8 (IL-8) release by budesonide.

Another well-characterized mechanism of theophylline is the antagonism at adenosine A1 as well as A2A and A2B receptors. In this context, endogenous adenosine released from neutrophils when acting via A2A receptors on neutrophils may serve to prevent their activation (Cronstein et al. 1985). When released from bronchial epithelial cells, endogenous adenosine acting via A2B receptors may increase ciliary motility and cystic fibrosis transmembrane conductance regulator (CFTR) activity by elevating cAMP (Rollins et al. 2008). As a corollary, antagonism by theophylline may favor proinflammatory effects in neutrophils or impair mucociliary functions of bronchial epithelial cells.

In clinical studies conducted with oral theophylline in COPD, this xanthine improved lung function to a variable extent, however, by primarily acting as a bronchodilator. For example, in a large trial the FEV_1 increase was comparable at day 1 and 12 weeks after daily theophylline intake (ZuWallack et al. 2001).

For theophylline, numerous side effects have been reported. Among them are arrhythmias, seizures, palpitations, tremor, nausea, headache, and insomnia. In addition, a considerable number of drug–drug interactions were described. For example, allopurinol, cimetidine, and erythromycin increase, whereas phenobarbital,

rifampicin, and phenytoin decrease theophylline plasma concentrations. In addition, smoking increases theophylline clearance (by inducing CYP1A2). All these interferences from concomitant drugs and smoking behavior complicate dosing, and, as a corollary, individual dose titration and plasma level monitoring are almost imperative measures in many cases (Boswell-Smith et al. 2006).

Even when theophylline was given at comparably low doses, as these were suggested for anti-inflammatory effects in asthmatics (Lim et al. 2001) or patients with COPD (Culpitt et al. 2002), about 13% of patients in a large trial of almost 4,000 patients had theophylline plasma concentrations in a range indicative of a higher risk for the occurrence of marked side effects (Ohta et al. 2004).

2.7.2 Other Oral PDE4 Inhibitors

Though the therapeutic index of second-generation PDE4 inhibitors was improved compared to rolipram, the development of many drugs including filaminast, lirimiast, piclamilast, CDP840, CI-1018, D-4418, IC485, L-826,141, SCH 351391, and V11294A has been discontinued because of a lack of efficacy, probably as the maximum tolerated dose was still in the lowest therapeutic or even subtherapeutic range (Giembycz 2008).

Apremilast (CC-10004, Celgene) (7) is another potent and selective, oral PDE4 inhibitor (IC_{50} at PDE4B of 33 nM, no selectivity among PDE4A–PDE4D subtypes) (Schafer et al. 2010), which initially may have been developed for asthma or COPD but recently has shown favorable efficacy in clinical trials on psoriasis and psoriatic arthritis. In preclinical studies, apremilast confirmed the expected *in vitro* anti-inflammatory profile of a PDE4 inhibitor. Yet more interestingly, apremilast showed efficacy in mouse models of psoriasis (Schafer et al. 2010) and rheumatoid arthritis (McCann et al. 2010).

In a small open-label, single-arm clinical study, 20 mg apremilast once daily over 4 weeks improved the psoriasis area and severity index (PASI) in 74% of patients with severe plaque-type psoriasis (Gottlieb et al. 2008). In those patients responding to apremilast with reduced epidermal thickness, the number of T-cells and CD11c-positive (dendritic) cells in dermis and epidermis, as well as total NOS in skin biopsies, was all suppressed. LPS-induced TNF- α release in *ex vivo* stimulated whole-blood samples taken 2 h after the first intake of apremilast showed 25–70% inhibition in 9 of 11 patients. In this context, half-maximum inhibition of LPS-induced TNF- α release in human whole blood *in vitro* by apremilast was at 316 nM and the maximum plasma concentration C_{max} in that clinical study was at 450 nM. Apparently, results from the *ex vivo* experiment were in line with expectations from comparing the potency in the *in vitro* experiment with the C_{max} value.

The promising clinical results were recently confirmed in larger randomized, double-blind, placebo-controlled trials in patients with moderate-to-severe psoriasis or psoriatic arthritis. In the plaque-type psoriasis trial, apremilast (30 mg BID, *p.o.*) met the primary end point of a 75% reduction of their PASI score (PASI-75) in 41%

of patients compared to 6% in the placebo group after 4 months treatment. Still, significant improvement was achieved with apremilast at the lower dose of 20 mg BID (29% of patients).

In a trial exploring the effects of the PDE4 inhibitor on patients with psoriatic arthritis, 43.5% of patients on apremilast at 20 mg BID, 35.8% of patients on apremilast at 40 mg OD, and 11.8% of patients on placebo achieved the primary end point of ACR20 (a composite end point indicating $\geq 20\%$ improvement of tender and swollen joint counts, pain, physical function, an inflammation laboratory marker, and overall disease activity, defined by the American College of Rheumatology, ACR) after only a 3-month treatment period.

Most common adverse events were nausea (18% [apremilast 30 mg BID] vs. 8% [placebo]), diarrhea (14% vs. 5%), headache, nasopharyngitis, and fatigue.

According to the clinical trials register, studies to explore efficacy of apremilast in an array of dermal indications such as contact dermatitis, gouty arthritis, rosacea, severe acne, uveitis, severe lichen planus, dermatomyositis, cutaneous sarcoidosis but also for other indications such as ankylosing spondylitis, and chronic prostatitis are currently ongoing or initiated.

2.7.3 Topical PDE4 Inhibitors

As an option to further improve their therapeutic index, PDE4 inhibitors, designed for topical administration either by the inhaled route for asthma and COPD, or as creams or ointments for atopic dermatitis and psoriasis, are under scrutiny by several companies (Fig. 2).

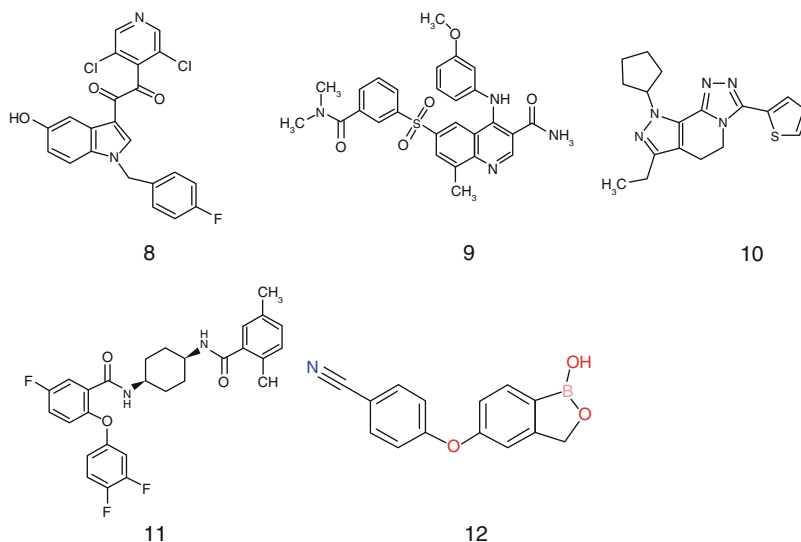


Fig. 2 Chemical structures of PDE4 inhibitors delivered by the topical route. (8) AWD 12-281; (9) GSK256066; (10) Tofimilast; (11) UK-500,001, (12) AN2728

The profile of an inhaled PDE4 inhibitor should comprise a long duration of action in the lungs along with rapid systemic metabolism resulting in low systemic exposure (AUC), high plasma protein binding, and low oral availability taking into account that after inhalation a considerable part of the compound is swallowed, altogether reducing the risk of systemic adverse events.

The benzamide AWD 12-281 (**8**, GSK842470, Elbion/GSK) was probably one of the first potent (IC_{50} at 3.5 nM) and selective PDE4 inhibitors specifically designed for an inhaled development (Draheim et al. 2004; Kuss et al. 2003; Gutke et al. 2005). AWD 12-281 exhibits rather low solubility, high plasma protein binding (>99%), and rapid clearance by phase II hepatic metabolism (apparently by glucuronidation perhaps to the hydroxyl group attached to the indole moiety). These properties may support extended lung retention, potentially fostering long duration of action in the lungs, yet allowing low systemic exposure following intratracheal administration as well as low oral bioavailability (Draheim et al. 2004). In this context, AWD 12-281 retained the inhibition of ovalbumin (OVA)-induced airway (bronchoalveolar lavage (BAL)) eosinophil influx in sensitized Brown–Norway (BN) rats when given at the (suboptimal) dose of $30 \mu\text{g kg}^{-1}$ intratracheally for up to 18 h before ovalbumin challenge, indicating a long duration of action (Kuss et al. 2003). In addition, AWD 12-281 administered intratracheally to rats reduced OVA-induced BAL eosinophil influx with ED_{50} of $7 \mu\text{g kg}^{-1}$ i.t. (BN rats) and LPS-induced BAL neutrophil influx with ED_{50} of $0.02 \mu\text{g kg}^{-1}$ i.t. (Lewis rats) (Kuss et al. 2003). At these dose levels, no relevant systemic exposure occurs. In consequence, the observed suppressive effects of AWD 12-281 on the lung inflammatory response to OVA or LPS are entirely attributed to the topical actions of the PDE4 inhibitor confined to the lungs. Remarkably, no signs of emesis or nausea from AWD 12-281 occurred in dogs following inhalation of up to 14 mg kg^{-1} per day over 4 weeks. Under these conditions, the systemic C_{max} was $1.5 \mu\text{M}$ (Kuss et al. 2003) that is close to the half-maximum inhibition of LPS-induced TNF- α release in human whole blood (EC_{50} of 900 nM, Kuss et al. 2003) or fMLP-induced neutrophil superoxide release in the presence of 80% autologous human plasma. AWD 12-281 has been in clinical development for asthma and COPD. Apparently results are not available in the public domain and further development may have been terminated.

GSK256066 (**9**, GlaxoSmithKline) is a more recent topical PDE4 inhibitor in clinical development for asthma, COPD, and allergic rhinitis. This quinoline analogue achieves a strikingly high potency to inhibit PDE4 in the low pM range (IC_{50} at 3.2 pM) with no difference between the PDE4A–PDE4D subtypes (Woodrow et al. 2009; Knowles et al. 2009). GSK256066 moderates early and late airway response (reflected by a loss in FEV_1) following allergen challenge in steroid-naïve atopic asthmatics after inhalation of $87.5 \mu\text{g}$ per day, once daily for 7 days (Singh et al. 2010). In this study, pharmacokinetic parameters were disclosed. Plasma area under the curve (AUC) at day 7 was only about 1.7-fold higher compared to day 1, and C_{max} (35 pM) were comparable. Given an extrapolated C_{max} unbound to proteins of 0.6 pM (plasma protein binding of 98.2%) and half-maximum inhibition of LPS-induced TNF- α release in human whole blood at

about 125 pM, the effects on extrapulmonary PDE4 or cell functions should be minor, with little impact on the favorable findings with inhaled GSK256066 on the airway allergen responses in that trial. As a corollary, the effects of inhaled GSK256066 in these asthmatics are explained by the compound's deposition in the lungs.

In a recently disclosed study (Laazar et al. 2010), inhaled GSK256066 at 25 or 87.5 $\mu\text{g kg}^{-1}$ per day was administered to patients with moderate COPD in a double-blind, placebo-controlled, randomized, 4-week trial. Primary end points were safety and efficacy; secondary end points included lung function, biomarkers of inflammation, and pharmacokinetics. In this trial, which was not powered to detect significant differences in lung function, inhaled GSK256066 at 25 or 87.5 $\mu\text{g kg}^{-1}$ per day reduced the lung residual volume (RV) by 141 and 367 ml, respectively (significant for the higher dose group). These findings may indicate a potential to reduce hyperinflation, considered a hallmark in COPD. Whether this observation translates into improved exercise capacity remains to be explored. Other lung volumes such as total lung capacity (TLC) or FRC followed more or less the findings for RV and there was a trend for (postbronchodilator) FEV₁ to dose-dependently increase (by 50 and 91 ml at 25 and 87.5 $\mu\text{g kg}^{-1}$ per day). In induced sputum, trends for a reduction in total proteins, IL-8, and myeloperoxidase (MPO) were reported. As requested for an inhaled compound, no relevant systemic PDE4-related adverse events were observed.

The solubility of GSK256066 is rather low ($<2 \mu\text{M}$), which along with one of the specificities of this quinoline that is slow reversal of [³H] GSK256066 binding from PDE4 (indicating a long drug-target residence time) may support long duration of action favoring once-daily administration.

The development of two earlier PDE4 inhibitors used in inhaled programs for COPD and asthma (tofigamist, **10**, and UK-500,001, **11**, both from Pfizer) (Danto et al. 2007a, b; Vestbo et al. 2009) has been discontinued for lacking or low efficacy in 6-week clinical trials (Vestbo et al. 2009).

In aggregate, while earlier endeavors with inhaled PDE4 inhibitors have been disappointing, initial experience with GSK256066 is more promising. On the basis of enzymatic, physicochemical, and pharmacokinetic parameters disclosed, GSK256066 was specifically designed for inhaled administration by integrating long pharmacological duration of action in the lungs with minimal systemic exposure. With curiosity new results of long-term studies with GSK256066 and comparable inhaled PDE4 inhibitors in asthma and COPD are awaited that may finally allow to assess whether there would be a position (and if affirmative where) of a well-designed inhaled PDE4 inhibitor in the management of these respiratory ailments.

The rationale behind developing PDE4 inhibitors specifically designed for topical administration to the skin in dermal indications was to improve the therapeutic index. While more than 30 years after Stawiski's seminal discovery that topically administered Ro 20-1724 reduces plaque size in psoriasis (Stawiski et al. 1979), no topical PDE4 inhibitor for this dermal indication has gained market access perhaps the most progressed and recent development is Anacor's AN2728

(12) (IC_{50} for PDE4 inhibition at 200 nM), a small molecule based on that company's proprietary boron chemistry platform (Akama et al. 2009; Nazarian and Weinberg 2009). In three placebo-controlled, randomized, double-blind phase II studies in patients with mild-to-moderate plaque-type psoriasis, AN2728 has shown significantly greater improvement in a specific score (Overall Target Plaque Severity Score, OTPSS) versus placebo after 6 weeks of twice-daily AN2728 (2%) ointment. On the basis of these promising findings, a phase III program with AN2728 will be embarked upon soon.

Along with orally given apremilast, AN2728 convincingly corroborates the pioneering findings of Stawiski and colleagues in 1979 that PDE4 inhibitors may alleviate psoriasis. The resurrection of an old concept provides hope that a PDE4 inhibitor specifically for psoriasis may finally reach the market.

3 Tolerability, Adverse Events, and Special Populations

3.1 *Rolipram*

The maximally tolerated dose of rolipram was 5 mg as a single oral dose, and 15 mg as a total daily dose, with nausea and vomiting as dose-limiting adverse reactions (Horowski and Sastre-Y-Hernandez 1985). In the small clinical studies to detect antidepressive effects (Zeller et al. 1984; Hebenstreit et al. 1989; Laux et al. 1988; Scott et al. 1991; Fleischhacker et al. 1992), but also in a later study exploring the potential of rolipram in multiple sclerosis (Bielekova et al. 2009), reports on the tolerability in the most frequently used doses (0.25–1 mg three time daily) are somewhat inconsistent with the majority of them indicating nausea (Laux et al. 1988; Hebenstreit et al. 1989; Scott et al. 1991). In the recent study on multiple sclerosis, where rolipram was dosed up to 3 mg three times daily, poor tolerability was reported (Bielekova et al. 2009).

In aggregate, inferiority of rolipram compared to common tricyclic antidepressives (Hebenstreit et al. 1989; Scott et al. 1991) along with a tolerability issue, when increasing the doses, may finally have contributed to abandoning the clinical development of this early PDE4 inhibitor.

3.2 *Ibudilast*

With therapeutic doses of ibudilast (total daily doses of 20 or 30 mg), the most common adverse events reported were anorexia and nausea as stated in the labeling information of Ketast[®] (Kyorin Pharmaceutical Co., Ltd., Japan). Gastrointestinal disorders such as gastrointestinal distress were described as primary adverse events in other studies (Ohtomo 1995; Shinohara et al. 2002).

When used with higher dose regimen (total oral dose of 40 or 60 mg per day), the most frequent adverse events observed with ibudilast were gastrointestinal symptoms (nausea and abdominal discomfort) (Feng et al. 2004; Kawasaki et al. 1992; Rolan et al. 2008), as well as hyperhidrosis and headache (Rolan et al. 2008). Headache and nausea were most frequently observed early in the multiday dosing period with ibudilast. In patients with multiple sclerosis, depression occurred more frequently as an adverse event in year 2 at 60 mg per day ibudilast than with placebo (Barkhof et al. 2010), although ibudilast has been reported to benefit in depression (Inoue and Harada 2008).

No consistent changes from baseline in individual laboratory parameters, vital signs, or electrocardiogram (ECG) were observed in healthy adult subjects with suprathreshold doses (60 mg for 14 days) (Rolan et al. 2008). According to the package insert of Ketas[®] (Kyorin Pharmaceutical Co., Ltd., Japan), of 14,968 Asian patients treated, elevated levels of liver enzymes were observed for aspartate aminotransferase (AST, 0.30%), alanine aminotransferase (ALT, 0.35%), and gamma-glutamyl transpeptidase (γ GT). Total bilirubin was increased in 0.36% of patients. Thrombocytopenia, anaemia, and leukopenia may occur as well.

3.3 Cilomilast

Gastrointestinal adverse events were the most frequent adverse events in an overall analysis of the cilomilast phase III program with COPD patients, with two or three times higher frequencies with cilomilast ($N = 2,088$) compared with placebo ($N = 1,408$) for nausea (16 and 5%), diarrhea (15 and 8%), abdominal pain (11 and 7%), vomiting (7 and 2%), and dyspepsia (7 and 2%) (Rennard et al. 2008). In a long-term safety study, the reported adverse event rates of nausea (44 and 14%), vomiting (21 and 8%), and diarrhea (33 and 13%) were nearly threefold higher with cilomilast (15 mg BID given as maintenance treatment for COPD over 12 months) than with placebo (study SB207499/121, quoted by Rennard et al. 2008), which is in agreement with tolerability results from a large efficiency study in Asians where at least a two times higher frequency of gastrointestinal adverse events arose with cilomilast than with placebo (Rennard et al. 2006).

Adverse events are generally transient and more prominent at the beginning of treatment with cilomilast. The frequency of undesirable gastrointestinal events was clearly dose-related. Nausea was usually mild to moderate and self-limiting in a 6-week dose-range study in COPD patients ($N = 424$) and occurred in 1, 1, 12, and 11% of patients randomized to receive placebo, low (5 mg), medium (10 mg), and high doses (15 mg) of cilomilast, respectively. Diarrhea was less common and occurred in 1, 2, 4, and 9% of the respective treatment groups (Compton et al. 2001).

Overall, gastrointestinal adverse events appear to occur more frequently with cilomilast than with roflumilast in comparable studies.

No differences in tolerability were observed between young and elderly healthy adults after single oral doses of cilomilast (10 mg) (Zussman et al. 2001a).

Cilomilast was not associated with clinically relevant changes in cardiac function, ECG, vital signs, or laboratory variables in healthy adults (Zussman et al. 2001a).

3.4 Roflumilast

Roflumilast displays an improved gastrointestinal side-effect profile compared with cilomilast, and vomiting is rare ($\leq 1\%$) with roflumilast treatment (Rabe et al. 2005). Frequent adverse events reported in pooled data from two large clinical trials with roflumilast in COPD patients were diarrhea (10% roflumilast vs. 3% placebo), weight decrease (7% roflumilast vs. 2% placebo), nausea (5% roflumilast vs. 1% placebo), and headache (5% roflumilast vs. 2% placebo) (Calverley et al. 2009). In addition to headache, undesirable effects also include dizziness, insomnia, and decreased appetite. Adverse events occur mainly within the first weeks of therapy and resolved during continued treatment.

Weight reduction was an adverse event with roflumilast treatment not previously reported for other selective PDE4 inhibitors. It is an event of interest, as reduced body weight of COPD patients is correlated with worse prognosis (Vestbo et al. 2006). The percentage of patients with weight reduction was highest in obese than in underweight patients (Calverley et al. 2009). The mean weight reduction was approximately 2 kg with roflumilast treatment after 1 year and mainly occurred during the first 6 months. Weight reduction was reversible after the cessation of treatment. Though weight decrease was more frequent in patients experiencing nausea, vomiting, or diarrhea, there does not seem to be a direct link to diarrhea. The exact cause of the observed weight decrease with roflumilast is unknown. Speculatively, it could have even been related to increased activity of patients who experience improvement of COPD symptoms (Zhang et al. 2009).

Roflumilast has no clinically relevant effect on vital signs, laboratory tests, and ECG parameters. In addition, the effects of roflumilast on cardiovascular function were studied in several clinical trials. By means of impedance cardiography (Bethke et al. 2001a; De Mey et al. 2006a), no effect on cardiac output was found in healthy volunteers. A thorough QT-study revealed no changes in ECG parameters (Hermann et al. 2006a). Also, in studies exploring interactions between the short- or long-acting β_2 -agonists salbutamol (Bethke et al. 2006) or formoterol (De Mey et al. 2006a) and roflumilast, there were no clinically meaningful changes in heart rate, mean systemic arterial blood pressure, or ECG parameters.

4 Pharmacokinetics

4.1 Rolipram

Rolipram has been investigated at oral doses of 0.25–1 mg given three times per day for treatment of depression.

Rolipram is rapidly absorbed after an oral administration. Absolute bioavailability was 73% (hepatic first-pass effect) (Krause et al. 1989). The maximum plasma concentration is reached after 15–45 min (Table 1).

The observed volume of distribution was small ($0.45 \text{ l}^{-1} \text{ kg}$), indicating lack of distribution to deep compartments.

Rolipram is extensively metabolized (Krause and Kühne 1993). The half-life of rolipram is short with approximately 3 h and the drug had to be administered three times a day (Krause et al. 1989) to achieve what was assumed being the therapeutically desired exposure. Rolipram is eliminated mainly by renal excretion (Krause et al. 1990). No pharmacokinetic differences have been observed between both enantiomers of rolipram (Krause et al. 1990).

Table 1 Pharmacokinetic parameters of PDE4 inhibitors in healthy adults following a single oral dose

Substance	Dose (mg) p.o.	T_{\max} (h)	C_{\max} (ng/ml)	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (ng h ml ⁻¹)	Reference
Rolipram ^a	1	0.4	34	3	250	Krause et al. (1989)
Ibudilast ^b	10	4	25	12	> 334	Uchida et al. (1985); labeling information KETAS [®] Capsules 10 mg
Tetomilast	50	2.75	5.7	24	140	Mallikaarjun et al. (2006)
Cilomilast	10	3	823	7	7,160	Zussman et al. (2001a)
Roflumilast	0.5	1	7.7	22.8	51	Lahu et al. (2010)
(Roflumilast N-oxide ^c)	N/A	4	10.9	24.7	529	Lahu et al. (2011)

^aracemic drug

^bAsian males; sustained release formulation

^cactive metabolite; p.o. oral

4.2 Ibudilast

Ibudilast is well absorbed after oral administration. Oral exposures adjusted for administered dose are higher in rats and humans than other species (Rolan et al. 2009). The maximum plasma concentration (C_{\max}) in humans is reached after 4–6 h (Rolan et al. 2008).

The plasma protein binding of ibudilast is high across species (Sanftner et al. 2009). Ibudilast exhibits rapid, extensive, and reversible CNS partitioning (Sanftner et al. 2009).

Ibudilast is metabolized by numerous cytochrome P450 isozymes and it is not predicted to be a clinically relevant inhibitor or inducer of CYP enzymes in vivo (Rolan et al. 2009).

Ibudilast is excreted primarily (60%) in urine with less than 0.01% recovered as unchanged drug (Rolan et al. 2008).

Human pharmacokinetics of ibudilast has been investigated in Asian and Caucasian healthy adults (Table 1) with single and multiple oral doses up to 30 mg BID (Uchida et al. 1985; Maeda et al. 1989; Rolan et al. 2008). After multiple oral doses, steady state of both ibudilast and the 6,7 dihydrodiol metabolite was reached after 2–3 days (BID regimen) (Rolan et al. 2008).

The C_{\max} after oral administration of ibudilast appeared to be slightly higher after the morning dose than after the evening dose (Rolan et al. 2008).

In contrast to the increased clearance observed in the rat with multiple oral doses of ibudilast, human pharmacokinetics seems to be invariant and non-inducible with repeated doses and gives no evidence of auto-induction or time-dependent pharmacokinetics (Rolan et al. 2008).

4.3 Tetomilast

Tetomilast was given up to 50 mg p.o., once daily. Following a single, oral dose of 50 mg to COPD patients C_{\max} was 5.7 ng ml⁻¹ (corresponding to 16 nM), the time to C_{\max} (t_{\max}) was 2.75 h, and the terminal half-life around 24 h ($t_{m1/2}$) allowing once-daily administration (Mallikaarjun et al. 2006). Tetomilast showed linear pharmacokinetics over a range of 25–100 mg p.o., single dose. Repeated administration resulted in a maximum twofold increase in exposure. Trough plasma concentrations were about 30–50% of the maximum. Absolute bioavailability following oral dosing was nearly 100% and absorption remained unaffected by food. The volume of distribution with about 180 ml kg⁻¹ indicates rather low distribution to tissues. Plasma protein binding was high with >99.5%. Following a single oral dose of radiolabeled tetomilast more than 90% of plasma radioactivity was attributed to the parent compound. Tetomilast may be metabolized by CYP1A1 and 1A2; however, the metabolite appears to be of minor importance.

4.4 Cilomilast

Cilomilast is rapidly and almost completely absorbed after oral administration (96%) (Zussman et al. 2001b). Maximum plasma concentration (C_{\max}) is reached within 3 h following oral administration (Zussman et al. 2001b).

Cilomilast is highly bound to plasma albumin (99%). Distribution of cilomilast into tissues is limited (volume of distribution 12 l) (Martina et al. 2006).

The drug is extensively metabolized by CYP 2C8 (Murdoch et al. 2004). Decyclopentylation, hydroxylation of the cyclopentyl ring, and glucuronidation are the main routes of metabolism of cilomilast (Zussman et al. 2000; Giembycz

2002). The metabolites are inactive (Giembycz 2002) with the main metabolite (SB 217493) being tenfold less potent than cilomilast as a PDE4 inhibitor (Giembycz 2002).

The plasma elimination half-life ($t_{m1/2}$) is short with approximately 7 h (Table 1) (Zussman et al. 2001b) and the drug must be administered twice daily.

Total and maximum exposure increase proportionally with the dose. Cilomilast shows similar pharmacokinetics following both single and repeated oral administrations though some accumulation (range: 1.04–1.18 with 4–20 mg) was observed (Zussman et al. 2001a). A pharmacokinetic steady state is reached after 3 days of continuous treatment.

Food significantly decreases the rate, but not the extent, of absorption, and administration with meals may reduce gastrointestinal adverse effects by reducing maximum exposure (Martina et al. 2006). Cilomilast plasma levels are not significantly affected by cigarette smoking (Kelly et al. 1999). Cilomilast has a low potential for pharmacokinetic or pharmacodynamic drug–drug interactions with commonly prescribed drugs including warfarin, digoxin, antacids, theophylline, prednisolone, and salbutamol (Giembycz 2001; Kelly et al. 2001; Murdoch et al. 1998, 2002, 2004; Zussman et al. 2001a, c). Drug–drug interaction studies of cilomilast with competitive CYP 2C8 substrates such as rosiglitazone have not been reported.

A small reduction in plasma half-life was noted following evening dosing compared to morning dosing in healthy adults taking cilomilast after eating a fat-rich meal (Zussman et al. 1999).

In renally impaired patients, the half-life and total plasma exposure (AUC) to free cilomilast increased progressively with severity of renal impairment (Zussman et al. 2002). This increase in cilomilast plasma exposure was paralleled by an increased incidence of adverse events.

Though cilomilast had a slightly higher peak plasma concentration in elderly patients and a prolonged elimination half-life compared with healthy young adults, these differences are not clinically significant (Zussman et al. 2001a).

4.5 Oglemilast

Oglemilast is well absorbed and has favorable pharmacokinetics permitting once-daily dosing. The highest doses tested in human single and multiple ascending dose studies were 24 mg. The pharmacokinetics was less than dose proportional at lower doses and higher doses. A single active metabolite, oglemilast N-oxide, was observed to have an exposure of nearly 25% of the parent compound and the same half-life as the parent compound. In vitro, the metabolite was found to be equipotent to the parent as inhibitor of PDE4. The half-life of oglemilast was 14–30 h. A shorter half-life of approximately 3 h and moderate bioavailability (>50%) was found in animal pharmacokinetic studies in mice, cynomolgus, and beagle but not in rats (9 h).

4.6 Roflumilast

Roflumilast is rapidly absorbed after oral administration with an absolute bioavailability of approximately 80% (Bethke and Lahu 2011). Roflumilast is metabolized in the liver to its major active metabolite, roflumilast N-oxide, mainly by the cytochrome P450 isozymes 3A4 and 1A2 (Fig. 3) (Bethke et al. 2007).

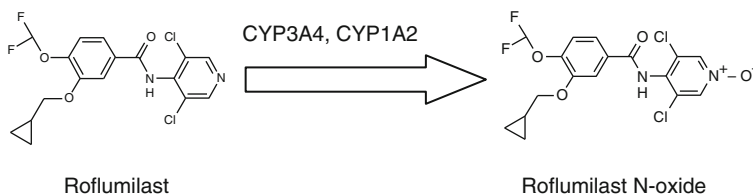


Fig. 3 Metabolism of roflumilast into its active metabolite, roflumilast N-oxide

The systemic exposure to roflumilast and roflumilast N-oxide increased proportionally with the administered dose (Bethke et al. 2002a, 2007). The maximum plasma concentration of roflumilast is reached after approximately 1 h and of roflumilast N-oxide after about 4 h (Table 1). Protein binding of both roflumilast and roflumilast N-oxide to human plasma is high (99 and 97%, respectively) (Bethke et al. 2007).

As a lipophilic substance, roflumilast exhibits a high volume of distribution, a property that makes roflumilast readily available to the peripheral tissue (Bethke and Lahu 2011). Other metabolites have also been observed in plasma as glucuronides.

Both roflumilast and roflumilast N-oxide are extensively metabolized and are excreted into urine with less than 1% unchanged parent drug (Bethke et al. 2002b).

The median half-lives of 22.8 and 24.7 h for roflumilast and roflumilast N-oxide are long and support once-daily oral dosing. Because of its long half-life and its systemic exposure, the active metabolite substantially contributes to the overall clinical efficacy of roflumilast. In fact, roflumilast N-oxide accounts for about 90% of the PDE4 inhibitor effect (Bethke et al. 2007).

Comparing the pharmacokinetics of morning and evening dosing, the maximum plasma concentration of roflumilast was marginally lower and the time to reach the peak concentration was slightly longer with evening dosing, while total exposures to parent drug and metabolite were unaffected (Bethke et al. 2010).

As roflumilast exhibits the unique property that both parent drug and metabolite considerably contribute to the overall pharmacologic effect, a measure was required to assess the total pharmacodynamic effect in conditions where their relative ratio might change and a potential rationale for dose adjustment would be needed. Considering that the intrinsic potency of roflumilast N-oxide is threefold lower compared to roflumilast, but the total plasma exposure of the metabolite is 10- to 12-fold higher with a threefold higher free fraction, the concept of total PDE4

inhibition (tPDE4i) (Fig. 4) has been developed (Hermann et al. 2006b). It permits relative quantitative comparisons of the pharmacodynamic effect on the basis of plasma concentrations, if extrinsic and intrinsic factors (e.g., drug–drug interactions, smoking, food or age, sex, and hepatic injury) might influence the CYP-dependent metabolism of roflumilast to the N-oxide (Hermann et al. 2006a, 2007b; von Richter et al. 2007; Lahu et al. 2011).

The tPDE4i concept was developed (Hermann et al. 2006b; Lahu et al. 2011) as an empirical construct to simplify the complex enzyme kinetics underlying the pharmacodynamic effect of roflumilast. Variables and fixed parameters that determine tPDE4i and are meaningful in a clinical pharmacology setting were included in this model. It was assumed that the contributions of roflumilast and roflumilast N-oxide are independent of each other and additive, which is true only for low concentrations. The inhibition may then be described as linear. The substrate (cAMP) concentration available for the enzyme PDE4 is assumed to be constant and sufficiently higher than the inhibitor concentrations. Consequently, the substrate concentration was not included in the model.

Roflumilast and roflumilast N-oxide have different affinities for the enzyme (potencies for PDE4 inhibition) as measured by the IC_{50} (inhibitory concentration 50%). The degree of PDE4 inhibition is indirectly proportional to the IC_{50} at a given inhibitor concentration and the model includes a correction term for IC_{50} .

The effective inhibitor concentrations c_i of roflumilast and roflumilast N-oxide, which may interact with PDE4, are determined by the fraction F_u of the total plasma concentration c_p , which is not bound to plasma proteins ($c_i = c_p \cdot F_u$). As the time to achieve the maximum plasma concentration (t_{max}) is different for roflumilast and roflumilast N-oxide, no single-point measurement is possible to investigate potential alterations of tPDE4i. The amount of drug in plasma, which is available for inhibition in the dosing interval, is described by plasma concentration (c_p)–time (t) curve. A quantitative measure is the area under the curve $AUC_{0-\tau}$ calculated as the integral $\int c_p dt$. An alignment of the fluctuating effective inhibitor concentration in time is possible by calculating moments such as $1/t \cdot AUC_{0-t}$ in the proposed model. Using the dosing interval τ as the time scale ($t = \tau$), the term $1/\tau \cdot (AUC_{0-\tau})$ will describe the aligned inhibitor concentrations of roflumilast or roflumilast N-oxide.

In summary, the tPDE4i by both active compounds was defined in (1) as the sum of the roflumilast (rof) and roflumilast N-oxide (rofNO) inhibitory effects given by the total in vivo exposure ($AUC_{0-\tau}$) at steady state during the dosing interval (τ), after accounting for substance differences in protein unbound fraction (F_u) and intrinsic activity (IC_{50}):

$$tPDE4i = \frac{AUC_{rof} \cdot Fu_{rof}}{IC_{50, rof} \cdot \tau} + \frac{AUC_{rofNO} \cdot Fu_{rofNO}}{IC_{50, rofNO} \cdot \tau}. \quad (1)$$

The $AUC_{0-\tau}$ was assessed as the experimental variable (calculated from $\int c_p dt$) in several clinical studies that compared normal with altered metabolic conditions.

Fixed parameters were the IC_{50} , F_u , and τ . The previously determined in vitro $IC_{50(rof)}$ of 0.7 nM and $IC_{50(rofNO)}$ of 2 nM were used for these calculations (Hatzelmann and Schudt 2001). The dosing interval τ was 24 h with a once-daily dosing. The free fractions of the PDE4 inhibitors were 1% for roflumilast and 3% for roflumilast N-oxide (Fig. 4) (Bethke et al. 2007).

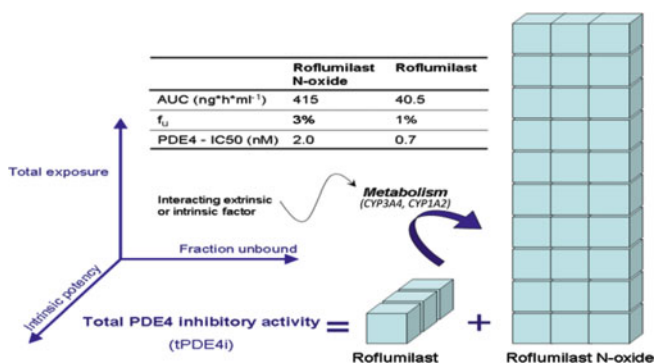


Fig. 4 The concept of total PDE4 inhibitory activity

No clinically relevant pharmacokinetic interactions were observed between roflumilast and commonly coprescribed drugs such as salbutamol (Bethke et al. 2006), formoterol, or budesonide (De Mey et al. 2006b; Hermann et al. 2007a).

No relevant drug interactions have been observed between roflumilast and inhibitors of its main metabolizing enzyme CYP 3A4 (ketoconazole, erythromycin, increase of tPDE4i by 9% each) nor with competitive substrates of CYP 3A4 (midazolam) (Lahu et al. 2008; Lahu et al. 2009; Nassr et al. 2007a). Coadministration of roflumilast with a potent CYP 1A2 inhibitor (fluvoxamine) increased the exposure of roflumilast by a factor of 2.6 and of roflumilast N-oxide by a factor of 1.5, respectively (von Richter et al. 2007). Coadministration of roflumilast and the typical cytochrome P450 enzyme inducer rifampicin reduced the exposure to roflumilast (C_{max} , AUC by 68 and 79%, respectively) and the N-oxide (C_{max} , AUC by 30 and 56%, respectively) (Nassr et al. 2009). Smoking is known to induce CYP 1A2 and roflumilast is (also) metabolized by this enzyme, but no effects of smoking status on the pharmacokinetics of roflumilast and roflumilast N-oxide have been observed in healthy adults (Bethke et al. 2001b), though population-pharmacokinetic data revealed a 20% decrease of exposure (Lahu et al. 2010).

The potential of roflumilast to interact with narrow therapeutic margin drugs such as digoxin (Eckermann et al. 2011), warfarin (McCracken et al. 2011), or theophylline (Böhmer et al. 2011) has also been studied. No clinically relevant pharmacokinetic interaction was found.

Subjects taking roflumilast in fed state have a significantly reduced maximum exposure (C_{max} decreased from 6.52 to 3.86 $\mu\text{g l}^{-1}$) to roflumilast, but the maximum plasma concentration of the active metabolite remains constant (Hauns et al. 2006). The time to reach the peak plasma concentration (T_{max} increase from 0.96

to 1.96 h) of roflumilast was prolonged with food intake. The tolerability of roflumilast was not altered by taking the drug with or without a meal or antacids (Nassr et al. 2007b). Roflumilast may consequently be taken independent of meals.

Slightly higher systemic exposures to roflumilast and its main metabolite were noted in females (45 and 43%, respectively), when compared with male subjects, but these pharmacokinetic differences were not considered clinically significant. Though tPDE4i was slightly higher in elderly compared with young adults, no dosage adjustment is recommended in elderly. Results of a population covariate analysis support that gender plays only a minor role (Lahu et al. 2011). Similarly, only a tendency for higher roflumilast and roflumilast N-oxide exposure in women was described from the interaction study with enoxacin (Lahu et al. 2011). As gender is a good predictor of CYP1A2 activity, higher levels of roflumilast and its metabolite were expected in female participants (Bebia et al. 2004). The total exposure to roflumilast and roflumilast N-oxide is increased with the degree of liver impairment (AUC 51%/24% higher in Child-Pugh A and 92%/41% higher in Child-Pugh B of roflumilast/roflumilast N-oxide, respectively). Concomitantly, the peak exposure is slightly increased with C_{\max} 3%/26% higher in Child-Pugh A and 26%/40% higher in Child-Pugh B of roflumilast/roflumilast N-oxide, respectively. The increase in exposure with mild hepatic impairment is not considered as clinically significant, but with moderate and severe hepatic impairment dose reduction of roflumilast should be considered (Hermann et al. 2007a).

Currently, it is recommended that roflumilast should be given with care in patients with mild hepatic impairment, whereas a dose of 500 μg cannot be administered in patients with moderate-to-severe liver impairment.

With severe renal impairment, systemic exposure to roflumilast and the metabolite is only slightly lower than in healthy adults and no dose adjustment is necessary (Bethke et al. 2002a).

5 Across Class Comparison and Discussion

A comparison of a few *in vitro* findings from PDE4 inhibitors that have been in clinical development for oral administration with the maximum total plasma concentrations in clinical trials may reveal some rather intriguing observations (Table 2).

The potency of the compounds to suppress PDE4 catalytic activity varied over a range of more than 1,000-fold from oglemilast to tetomilast (ibudilast as a rather nonselective PDE inhibitor aside). No substantial difference with the known exception of cilomilast showing some PDE4D selectivity was found for inhibition of PDE4B1 compared to PDE4D3 catalytic activity for all compounds including rolipram that, however, was associated with side effects such as nausea and vomiting resulting in an unfavorable therapeutic index. Two cellular assays were selected that may allow direct comparison of *in vitro* potencies of the PDE4 inhibitors with their peak plasma levels in the clinical studies (irrespective of plasma

Table 2 Comparison of enzymatic and cellular findings with human C_{max} for oral PDE4 inhibitors

Oral PDE4 inhibitor	PDE4B IC ₅₀ (nM)	PDE4D IC ₅₀ (nM)	Selectivity Fold	LPS/TNF wb IC ₃₀ (nM)	PMN/ROS (plasma) IC ₃₅ (nM)	Ratio [#] fold	C_{max} (human) nM	Clinical dose
Rolipram	230	120	1,000	500	90	0.2	≈40	0.75 mg TID, p.o., d28 ^a
Ibudilast	3,300 ^b	3,700 ^b	0.7 (PDE10)	ND	ND	ND	260	30 mg BID, p.o., d16 ^c
Tetomilast	380	ND	×8 (PDE3)	30,200	9,120	0.3	16	50 mg p.o., single dose ^d
Cilomilast	140	20	150	5,000	1,000	0.2	7,800	15 mg BID, p.o., d9 ^e
Oglemilast	0.3	ND	>1,000	90	1,820	20	Unknown	800 µg OD
Roflumilast	0.7	0.4	>1,000	50	90	1.8	15	0.5 mg OD, p.o., d7 ^f
Roflumilast N-oxide	1.5	0.8	>1,000	50	110	2.2	52	
Apremilast	33/49 ^g	ND/30 ^g	>300	316	912	2.8	450	20 mg OD, p.o., d29 ^h

Data for inhibition of PDE4B1 and PDE4D3 catalytic activity, PDE isoenzyme selectivity, LPS-induced TNF- α release in human whole blood (LPS/TNF wb), and fMLP-induced superoxide release from human PMN in the presence of 80% human autologous plasma (PMN/ROS (Plasma)) were generated by one of the authors (A.H.) according to the methods described in Hatzelmann and Schudt (2001), with the exception of Ibudilast, taken from Gibson et al. (2006) and apremilast (PDE4B2 and PDE4D3, inhibition of catalytic activity) taken from Schafer et al. (2010). For the LPS/TNF wb and PMN/ROS (plasma) assay, IC₃₀ and IC₃₅ refer to half-maximum inhibition given that the efficacy achieved with a PDE4 inhibitor was 60 and 70% inhibition, respectively. Plasma total C_{max} values at the indicated clinical doses are obtained from the references listed below. ND is not done. [#]Ratio is the IC₃₅ (PMN/ROS, plasma) divided by IC₃₀ (LPS/TNF wb)

^aLaux et al. (1988)

^bGibson et al. (2006)

^cRolan et al. (2008)

^dMallikaarjun et al. (2006)

^eZaassman et al. (2001a)

^fBethke et al. (2007)

^gSchafer et al. (2010)

^hGottlieb et al. (2008)

protein binding). These are inhibition of LPS-induced TNF- α release from human whole blood and inhibition of superoxide release from fMLP-stimulated human polymorphonuclear cells (PMN) in the presence of 80% human autologous plasma (Hatzelmann and Schudt 2001). Comparing the potency of the different PDE4 inhibitors in the two assays reveals a group of compounds (Rolipram, Tetomilast, and Cilomilast) that were more potent in suppressing neutrophil superoxide release compared to LPS-induced TNF- α release from human whole blood. For another group of compounds, encompassing roflumilast (launched in EU for severe COPD) and apremilast (with promising results from clinical studies in psoriasis) but also oglemilast, their potency to reduce neutrophil superoxide formation (80% plasma) was inferior to that involved with diminishing LPS-induced TNF- α release in the whole-blood assay. For rolipram following repeated dosing of 750 μg three times daily, the C_{max} of about 40 nM was closer to the half-maximum inhibition of fMLP-stimulated superoxide release from PMN (90 nM) than to half-maximum inhibition of LPS-induced TNF- α release in human whole blood (500 nM). On the other hand, for roflumilast N-oxide the C_{max} (52 nM) after repeated dosing was in the range of half-maximum TNF- α reduction in the whole-blood assay (50 nM) although below the half-maximum inhibition of superoxide generation from PMN (110 nM). In this respect, apremilast showed a rather comparable behavior to roflumilast. For cilomilast, however, while as for roflumilast N-oxide and apremilast, plasma C_{max} after repeated dosing was well in the range of the compound's potencies in the two *in vitro* assays, at the peak plasma levels of 7.8 μM cilomilast neutrophil superoxide release (80% plasma) may have been more completely attenuated than LPS-induced TNF- α formation in the whole-blood assay. One may reiterate here the earlier observation that inhibition of some neutrophil functions was considered to correlate to emesis (Barnette et al. 1998).

Curiously, for tetomilast peak plasma concentrations reached after a single oral dose of 50 mg as given in a communication by Otsuka (Mallikaarjun et al. 2006) was much less than it would have been required for inhibition of the above-mentioned two functional readouts (50 mg once daily was given in the clinical studies). While repeated dosing may bear the potential of somewhat increasing C_{max} and total exposure, such an effect may not be expected to fully account for the difference.

Also for ibudilast, peak plasma levels following repeated oral dosing of 30 mg BID were below IC_{50} values for PDE4 inhibition, in particular considering a plasma protein binding of $\geq 95\%$ (Sanftner et al. 2009), resulting in peak plasma levels unbound to protein of ≤ 13 nM (according to Kyorin's packing insert ibudilast is recommended three times daily at 10 mg per dose).

As an unselective PDE inhibitor with some preference to inhibit PDE3 (IC_{50} of 110 μM) over PDE4B (IC_{50} of 660 μM), theophylline is not listed in the table. However, this xanthine represents an example for a compound that was long assumed to exert clinical effects also by PDE4 inhibition. But finally it does not as reflected by some simple extrapolations from plasma levels. The recommended plasma concentrations for theophylline are well-known as between 5 and 20 mg l^{-1} corresponding to 28–112 μM total. With a plasma protein binding of about 60%, the concentrations unbound to proteins are between 11 and 44 μM . Over this

concentration range, PDE4 is inhibited <10% and it appears unlikely, therefore, that inhibition of the enzyme's catalytic activity contributes to clinical effects. On the other hand, an approximately 20–35% inhibition of PDE3 may be expected between 11 and 44 μM that in general is perceived to at least support the acute bronchodilatory effects found with theophylline.

Theophylline is an antagonist to adenosine A1, A2A, and A2B receptors at K_i of 1–10 μM (Fozard and Hannon 1999), which is below the therapeutic range, and as a corollary adenosine receptor antagonism is considered to contribute to efficacy and adverse events.

As discussed above, theophylline suppresses PI3K δ , sensitized by oxidative stress (EC_{50} of around 1 μM and maximum inhibition of 60% at $\geq 10 \mu\text{M}$) translating into protection of HDAC2 activity over a concentration range of 1–10 μM theophylline (To et al. 2010; Barnes 2009), which is close or below the therapeutic plasma levels for this xanthine. Clearly, such a mechanism may more likely contribute to clinical effects of theophylline than PDE4 inhibition.

In contrast, the standard clinical dose of roflumilast at 500 μg OD results in plasma levels of 1–2 nM roflumilast N-oxide (unbound to proteins) maintained over the 24 h dosing interval, which correspond to an extrapolated 50–60% inhibition of PDE4 translating into 20–40% inhibition of cellular functions (see Hatzelmann et al. 2010 for a recent review). Thus, for roflumilast one may assume that clinical efficacy is fully attributed to highly potent and selective PDE4 inhibition. Clearly, this pharmacokinetic profile with rather stable plasma levels in the once-daily dosing regimen represents an asset of roflumilast that distinguishes this PDE4 inhibitor from earlier competitors (Bethke et al. 2007; Hatzelmann et al. 2010). This is further illustrated in Fig. 5 where the pharmacokinetic profile following repeated dosing of roflumilast at the clinical dose of 500 μg OD was recalculated to the extent of PDE4B1 and PDE4D3 inhibition for the (1) sum of roflumilast and its active metabolite and for (2) roflumilast and (3) roflumilast N-oxide alone.

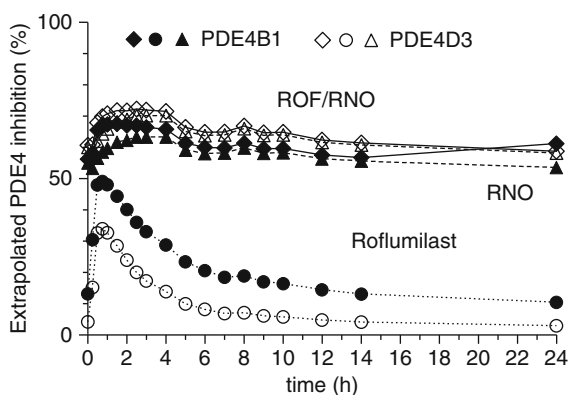


Fig. 5 Extrapolated extent of PDE4B and D inhibition following repeated dosing of roflumilast (500 μg OD) based on plasma levels. Circles, roflumilast; triangles, roflumilast N-oxide; diamonds, sum of roflumilast and roflumilast N-oxide

On the basis of this profile, it is obvious again that the active metabolite is considered to largely account for the clinical efficacy of roflumilast documented in COPD patients (Calverley et al. 2009; Fabbri et al. 2009).

Roflumilast is unique in its class with both metabolite and parent drug contributing to PDE4 inhibition. The resulting overall pharmacodynamic effect is maintained over the dosing interval, irrespective of factors influencing the metabolic conversion of roflumilast to its N-oxide. This adds to the predictability of efficacy and safety in COPD patients by avoiding therapeutic failure or adverse drug reactions in case of concomitant medication or comorbidities, because the effect of increased or decreased metabolism will be outbalanced. The parallel metabolic pathways of roflumilast by CYP 3A4 and 1A2 additionally compensate effects of coadministration of roflumilast and enzyme inhibitors. As confirmed by drug–drug interaction studies, roflumilast reveals a common interaction profile on the basis of its CYP 3A4 and 1A2 metabolism. When given together with enoxacin, cimetidin, fluvoxamin, and rifampicin, the efficacy and safety needs to be clinically followed.

The striking difference and progress in the pharmacokinetic profiles from the early rolipram to roflumilast is schematically illustrated by comparing plasma levels over time following repeated administration of their clinical doses in humans (Fig. 6).

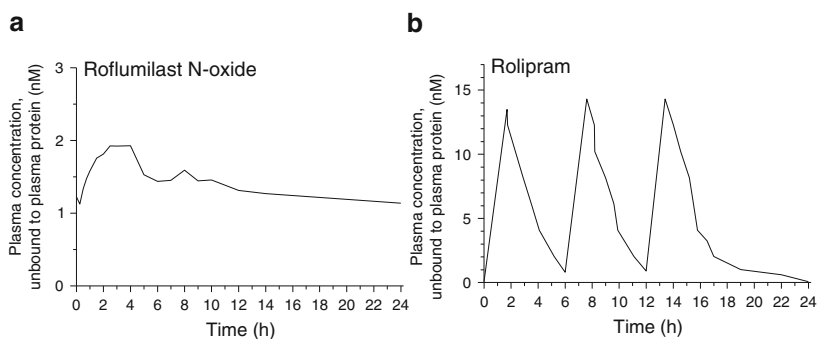


Fig. 6 Plasma levels over time (unbound to plasma protein) following repeated oral administration of roflumilast (500 μ g, once daily) (a) or rolipram (750 μ g, three times daily) (b). In (a), plasma levels for roflumilast N-oxide are shown, given that it accounts for >90% of overall PDE4 inhibition. In (b), plasma levels are adapted from Krause et al. 1989 considering plasma protein binding of 85%

For roflumilast N-oxide, plasma levels unbound to protein remain in a rather narrow range over an entire 24 h dosing interval. In contrast, rolipram plasma levels are fluctuating by more than 15-fold between trough and peak concentrations. While evaluating these profiles, it is intriguing that, in general, unfolding anti-inflammatory effects of PDE4 inhibitors in the clinics likely require a rather constant level of PDE4 inhibition over a dosing interval. Side effects (such as nausea) are perceived as occurring rapidly, yet being rather transient in nature. One

may propose the idea that the rather smooth pharmacokinetic profile, specifically the lack of sharp C_{\max} peaks obtained following repeated dosing of roflumilast in humans, contributes to its favorable tolerability versus earlier PDE4 inhibitors such as rolipram. In this respect, pharmacokinetics is one important determinant of overall efficacy versus side effects, in addition to the ratio of the inhibition of fMLP-stimulated superoxide release from neutrophils (80% plasma) versus LPS-induced TNF- α release in human whole blood as discussed before.

The discussions above are based on many extrapolations from plasma levels to cellular effects (and even those in humans), which certainly have their limitations. For example, concentrations of compounds at the target cells remain unknown. They may perhaps be close to those in the plasma, but there are no studies proving this concept in detail. Results of the *in vitro* cellular assays with whole blood or plasma may vary with different experimental conditions or stimulus concentrations. Notwithstanding this, one interesting observation remains that the two compounds, which perhaps most convincingly showed clinical efficacy (roflumilast and apremilast), are among those with clinical plasma levels corresponding to substantial inhibition of PDE4 and cellular functions.

On the basis of cellular and specifically animal studies, the therapeutic potential of PDE4 inhibitors may be wide ranging from COPD and asthma to idiopathic pulmonary fibrosis, inflammatory bowel disease, psoriasis, and atopic dermatitis, then rheumatoid arthritis, osteoporosis to B-cell chronic lymphocytic leukemia (B-CLL), acute promyelocytic leukemia, and even Alzheimer's and cognitive deficits to name but a few. What could be the strategies for design of PDE4 inhibitors with an even improved therapeutic index compared with those that currently are rather promising?

One strategy may be topical administration when possible, such as for respiratory or skin disorders, by designing compounds with a high local, yet very limited, systemic exposure, mostly based on physical chemistry and pharmacokinetic optimization. As some of the ailments with prominent local manifestations bear critical systemic components (such as in COPD or psoriasis), these approaches may eventually reveal to be of limited potential as based on their design the compounds may not address these systemic components of the disorder.

Another strategy that was exploited for cilomilast, and perhaps tetomilast, has been to design compounds that based on their pKa are cationic or anionic at physiological pH. These charged species may be expected to bear low passive penetration over the blood-brain barrier to limit CNS-related adverse events. However, this approach may not reduce the appearance of emesis because the area postrema is located outside the blood-brain barrier. In addition, as observed with cilomilast and tetomilast, compounds being anionic at physiological pH may show a high plasma protein binding.

A third approach has been to generate PDE4B-selective compounds, following the optimism generated by results from the PDE4B- versus PDE4D-deficient mice that PDE4B accounts for many anti-inflammatory effects, whereas PDE4D may be related to emesis. While generating subtype selectivity for PDE4B appears a challenging endeavor for the medicinal chemist, over the past few years, evidence

has accumulated that PDE4D as well is intimately related to many inflammatory and remodeling processes, and PDE4B to some CNS effects. In summary, it is currently unclear whether an improved therapeutic index can be expected from a selective PDE4B inhibitor, or if, with such a compound, a possible gain in safety is counterbalanced by a loss in anti-inflammatory effects. This reasoning has tempered somewhat the enthusiasm of PDE4B-selective inhibitors.

Perhaps the most exciting approach emerged with the allosteric PDE4 modulators interacting also with the Upstream Conserved Regulatory domain-2 (UCR2) (Burgin et al. 2010). If the promise of a large therapeutic index between improved cognitive function and the emesis-correlate in mice for the PDE4D-selective, allosteric inhibitors is translated into clinics, it may in fact herald the dawning of a novel era in PDE4 research.

Finally, there have been attempts to design dual-selective, for example, PDE3/4, PDE4/7, or PDE1/4 inhibitors based on reasoning that synergisms between PDE4 inhibition and that of the other enzyme shown on a cellular level may allow to reduce the extent of PDE4 inhibition required to attain similar therapeutic effects (Giembycz 2008). One should consider that inhibition of the other PDE family may carry a risk of additional adverse effects. Several attempts in the past have been abandoned.

In conclusion, optimizing pharmacokinetic characteristics has been critical to the design of clinically useful PDE4 inhibitors.

References

- Agusti JA, Soriano JB (2008) COPD as a systemic disease. *COPD* 5:133–138
- Akama T, Baker SJ, Zhang YK, Hernandez V, Zhou H, Sanders V, Freund Y, Maples KR, Plattner JJ (2009) Discovery and structure-activity study of a novel benzoxaborole anti-inflammatory agent (AN2728) for the potential topical treatment of psoriasis and atopic dermatitis. *Bioorg Med Chem Lett* 19:2129–2132
- Ariga M, Neitzert B, Nakae S, Mottin G, Bertrand C, Pruniaux MP, Jin SL, Conti M (2004) Nonredundant function of phosphodiesterases 4D and 4B in neutrophil recruitment to the site of inflammation. *J Immunol* 173:7531–7538
- Barkhof F, Hulst HE, Drulovic J, Uitdehaag BM, Matsuda K, Landin R, MN166-001 Investigators (2010) Ibudilast in relapsing-remitting multiple sclerosis: a neuroprotectant? *Neurology* 74:1033–1040
- Barnes PJ, Rennard SI (2008) Pathophysiology of COPD. In: Barnes PJ, Drazen J, Rennard SI, Thomson N (eds) *Asthma and COPD, basic mechanisms and clinical management*. Academic, Amsterdam, pp 425–442
- Barnes PJ (2009) Role of HDAC2 in the pathophysiology of COPD. *Annu Rev Physiol* 71: 451–464
- Barnes PJ, Celli BR (2009) Systemic manifestations and comorbidities of COPD. *Eur Respir J* 33:1165–1185
- Barnette MS, Christensen SB, Essayan DM, Gours M, Prabhakar U, Rush JA, Kagey-Sobottka A, Torphy TJ (1998) SB207499 (Ariflo), a potent and selective second generation phosphodiesterase 4 inhibitor: In vitro anti-inflammatory actions. *J Pharmacol Exp Ther* 284:420–426

- Bebia Z, Buch SC, Wilson JW et al (2004) Bioequivalence revisited: influence of age and sex on CYP enzymes. *Clin Pharmacol Ther* 76:618–627
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Bethke Th, Hartmann M, Baumgartner A, Eichberger C, Hauns B, Wurst W (2001a) The new PDE4 inhibitor roflumilast does not influence cardiovascular function. *Am J Respir Crit Care Med* 163(5):A431
- Bethke Th, Hünne Meyer A, Hauns B, Diedrich A, Baumgartner A, Eichberger C, Drollmann A, Hartmann M, Neuhäuser M, David M, Zech K, Wurst W (2001b) Smoking has no effect on the pharmacokinetics of roflumilast, a new, orally active, selective PDE4 inhibitor. *Eur Respir J Suppl* 18(33):156s, P1061
- Bethke TD, Hauns B, Zech K, David M, Wurst W (2002a) Dose linearity of roflumilast, a new selective PDE4 inhibitor. *Am J Respir Crit Care Med* 165:A595
- Bethke T, Hartmann M, Zech K, David M, Weimar C, Wurst W (2002b) No dose adjustment of roflumilast in patients with severe renal impairment. *Am J Respir Crit Care Med* 165: A594
- Bethke TD, Giessmann T, Westphal K, Weinbrenner A, Hauns B, Hauschke D, David M, Lahu G, Zech K, Hermann R, Siegmund W (2006) Roflumilast, a once-daily oral phosphodiesterase 4 inhibitor, lacks relevant pharmacokinetic interactions with inhaled salbutamol when co-administered in healthy subjects. *Int J Clin Pharmacol Ther* 44:572–579
- Bethke TD, Böhmer GM, Hermann R, Hauns B, Fux R, Mörike K, David M, Knoerzer D, Wurst W, Gleiter CH (2007) Dose-proportional intraindividual single- and repeated-dose pharmacokinetics of roflumilast, an oral, once-daily phosphodiesterase 4 inhibitor. *J Clin Pharmacol* 47:26–36
- Bethke TD, Lahu G (2011) High absolute bioavailability of the new oral phosphodiesterase 4 inhibitor roflumilast. *Int J Clin Pharmacol Ther* 49:51–58
- Bethke TD, Hünne Meyer A, Lahu G, Lemmer B (2010) Chronopharmacology of roflumilast: a comparative pharmacokinetic study of morning vs. evening administration in healthy adults. *Chronobiol Int* 27:1843–1853
- Bielekova B, Richert N, Howard T, Packer AN, Blevins G, Ohayon J, McFarland HF, Stürzebecher CS, Martin R (2009) Treatment with the phosphodiesterase type-4 inhibitor rolipram fails to inhibit blood–brain barrier disruption in multiple sclerosis. *Mult Scler* 15:1206–1214
- Böhmer G, Hünne Meyer A, Lahu G, Gleiter CH, Bethke TD (2011) Theophylline and in healthy adults. *Br J Clin Pharmacol* (in press)
- Boswell-Smith V, Cazzola M, Page CP (2006) Are phosphodiesterase 4 inhibitors just more theophylline? *J Allergy Clin Immunol* 117:1237–1243
- Boswell-Smith V, Spina D (2007) PDE4 inhibitors as potential therapeutic agents in the treatment of COPD-focus on roflumilast. *Int J Chron Obstruct Pulmon Dis* 2:121–129
- Bundschuh DS, Eltze M, Barsig J, Wollin L, Hatzelmann A, Beume R (2001) In vivo efficacy in airway disease models of roflumilast, a novel orally active PDE4 inhibitor. *J Pharmacol Exp Ther* 297:280–290
- Burgin AB, Magnusson OT, Singh J, Witte P, Staker BL, Björnsson JM, Thorsteinsdóttir M, Hrafnadóttir S, Hagen T, Kiselyov AS, Stewart LJ, Gurney ME (2010) Design of phosphodiesterase 4D (PDE4D) allosteric modulators for enhancing cognition with improved safety. *Nat Biotechnol* 28:63–70
- Calverley PM, Rabe KF, Goehring UM, Kristiansen S, Fabbri LM, Martinez FJ, M2-124 and M2-125 study groups (2009) Roflumilast in symptomatic chronic obstructive pulmonary disease: two randomised clinical trials. *Lancet* 374:685–694
- Calverley PMA, Martinez FJ, Goehring UM, Rennard SI (2010) Defining patient populations in COPD: experience with roflumilast. Presented at: 7th International Multidisciplinary Conference on Chronic Obstructive Pulmonary Disease (COPD7), Birmingham, UK, 30 June–2 July 2010 (Poster 48)

- Cho Y, Crichlow GV, Vermeire JJ, Leng L, Du X, Hodsdon ME, Bucala R, Cappello M, Gross M, Gaeta F, Johnson K, Lolis EJ (2010) Allosteric inhibition of macrophage migration inhibitory factor revealed by ibudilast. *Proc Natl Acad Sci USA* 107:11313–11318
- Choi SH, Sakamoto T, Fukutomi O, Inagaki N, Matsuura N, Nagai H, Koda A (1989) Pharmacological study of phospholipase A2-induced histamine release from rat peritoneal mast cells. *J Pharmacobiodyn* 12:517–522
- Compton CH, Gubb J, Nieman R, Edelson J, Amit O, Bakst A, Ayers JG, Creemers JP, Schultze-Werninghaus G, Brabilla C, Barnes NC (2001) Cilomilast, a selective phosphodiesterase-4 inhibitor for treatment of patients with chronic obstructive pulmonary disease: a randomised, dose-ranging study. *Lancet* 358:265–270
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- Cosio BG, Iglesias A, Rios A, Noguera A, Sala E, Ito K, Barnes PJ, Agusti A (2009) Low-dose theophylline enhances the anti-inflammatory effects of steroids during exacerbations of COPD. *Thorax* 64:424–429
- Cronstein BN, Rosenstein ED, Kramer SB, Weissmann G, Hirschhorn R (1985) Adenosine; a physiologic modulator of superoxide anion generation by human neutrophils: Adenosine acts via A2 receptor on neutrophils. *J Immunol* 135:1366–1371
- Culpitt SV, de Matos C, Russell RE, Rogers DLE, DF BPJ (2002) Effect of theophylline on induced sputum inflammatory indices and neutrophil chemotaxis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 165:1371–1376
- Danto S, Wei GC, Gill J et al (2007a) A randomized, double-blind, parallel group, six-week study of the efficacy and safety of tofomilast dry powder for inhalation (DPI) in adults with persistent asthma. *Am J Respir Crit Care Med* 175:A485
- Danto S, Wei GC, Gill J et al (2007b) A randomized, double-blind, parallel group, six-week study of the efficacy and safety of tofomilast dry powder for inhalation (DPI) in adults with COPD. *Am J Respir Crit Care Med* 175:A131
- De Mey C, Hauns B, Knoerzer D, Grossmann M, Haverkamp W, Bethke TD, Hermann R (2006a) Roflumilast does not potentiate tachycardia associated with formoterol. *Proc Am Thorac Soc* 3:A112
- De Mey C, Hauns B, Lahu G, Reutter F, Knoerzer D, Grossmann M, Haverkamp W, Bethke TD, Hermann R (2006b) Repeated-dose co-administration of roflumilast and formoterol does not alter the pharmacokinetics of either drug. *Proc Am Thorac Soc* 3:A113
- Draheim R, Egerland U, Rundfeldt C (2004) Anti-inflammatory potential of the selective phosphodiesterase 4 inhibitor N-(3, 5-dichloro-pyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide (AWD 12–281), in human cell preparations. *J Pharmacol Exp Ther* 308:555–663
- Eckermann G, Lahu G, Nassr N, Bethke TD (2011) Absence of pharmacokinetic interaction between roflumilast and digoxin in healthy adults. *J Clin Pharmacol* (in press)
- Engelstaetter R, Wingertzahn M, Schmid-Wirlitsch C, Bredenbroeker D, Leichtl S, Wurst W (2002). Roflumilast, an investigational oral, once daily, phosphodiesterase-4 inhibitor, did not exhibit acute bronchodilatory activity after a single dose. American College of Allergy, Asthma and Immunology (ACAAI), Annual Conference, Boston 2004
- Fabbri LM, Calverley PM, Izquierdo-Alonso JL, Bundschuh DS, Brose M, Martinez FJ, Rabe KF (2009) Roflumilast in moderate-to-severe chronic obstructive pulmonary disease treated with long-acting bronchodilators: two randomised clinical trials. *Lancet* 374:695–703
- Feng J, Misu T, Fujihara K, Sakoda S, Nakatsuji Y, Fukaura H, Kikuchi S, Tashiro K, Suzumura A, Ishii N, Sugamura K, Nakashima I, Itoyama Y (2004) Ibudilast, a nonselective phosphodiesterase inhibitor, regulates Th1/Th2 balance of NKT cell subset in multiple sclerosis. *Mult Scler* 10:494–498
- Fleischhacker WW, Hinterhuber H, Bauer H, Pflug B, Berner P, Simhandl C, Wolf R, Gerlach W, Jaklitsch H, Sastre-y-Hernández M et al (1992) A multicenter double-blind study of three

- different doses of the new cAMP-phosphodiesterase inhibitor rolipram in patients with major depressive disorder. *Neuropsychobiology* 26:59–64
- Ford PA, Durham AL, Russell RE, Gordon F, Adcock IM, Barnes PJ (2010) Treatment effects of low-dose theophylline combined with an inhaled corticosteroid in COPD. *Chest* 137:1338–1344
- Fozard JR, Hannon JP (1999) Adenosine receptor ligands: potential as therapeutic agents in asthma and COPD. *Pulm Pharmacol Ther* 12:111–114
- Gamble E, Grootendorst DC, Brightling CE, Troy S, Qiu Y, Zhu J, Parker D, Matin D, Majumdar S, Vignola AM, Kroegel C, Morell F, Hansel TT, Rennard SI, Compton C, Amit O, Tat T, Edelson J, Pavord ID, Rabe KF, Barnes NC, Jeffery PK (2003) Antiinflammatory effects of the phosphodiesterase-4 inhibitor cilomilast (Ariflo) in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 168:976–982
- Gibson LC, Hastings SF, McPhee I, Clayton RA, Darroch CE, Mackenzie A, Mackenzie FL, Nagasawa M, Stevens PA, Mackenzie SJ (2006) The inhibitory profile of Ibudilast against the human phosphodiesterase enzyme family. *Eur J Pharmacol* 538:39–42
- Giembycz MA (2001) Cilomilast: a second generation phosphodiesterase 4 inhibitor for asthma and chronic obstructive pulmonary disease. *Expert Opin Investig Drugs* 10:1361–1379
- Giembycz MA (2002) Development status of second generation PDE4 inhibitors for asthma and COPD: the story so far. *Monaldi Arch Chest Dis* 57:48–64
- Giembycz MA (2008) Can the anti-inflammatory potential of PDE4 inhibitors be realized: guarded optimism or wishful thinking? *Br J Pharmacol* 155:288–290
- Gottlieb AB, Strober B, Krueger JG, Rohane P, Zeldis JB, Hu CC, Kipnis C (2008) An open-label, single-arm pilot study in patients with severe plaque-type psoriasis treated with an oral anti-inflammatory agent, apremilast. *Curr Med Res Opin* 24:1529–1538
- Grootendorst DC, Gauw SA, Baan R, Kelly J, Murdoch RD, Sterk PJ, Rabe KF (2003) Does a single dose of the phosphodiesterase 4 inhibitor, cilomilast (15 mg), induce bronchodilation in patients with chronic obstructive pulmonary disease? *Pulm Pharmacol Ther* 16(115–120):2003
- Grootendorst DC, Gauw SA, Verhoosel RM, Sterk PJ, Hospers JJ, Bredenbröker D, Bethke TD, Hiemstra PS, Rabe KF (2007) Reduction in sputum neutrophil and eosinophil numbers by the PDE4 inhibitor roflumilast in patients with COPD. *Thorax* 62:1081–1087
- Gutke HJ, Guse JH, Khobzaoui M, Renukappa-Gutke T, Burnet M (2005) AWD-12-281 (inhaled) (elbion/GlaxoSmithKline). *Curr Opin Investig Drugs* 6:1149–1158
- Hanania NA, Brose M, Larsson T, Rabe KF (2010) Efficacy of roflumilast in patients receiving concomitant treatments for chronic obstructive pulmonary disease over 12 months. *Am J Respir Crit Care Med* 181:4435
- Hatzelmann A, Schudt C (2001) Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *J Pharmacol Exp Ther* 297:267–279
- Hatzelmann A, Morcillo EC, Lungarella G, Adnot S, Shahin S, Rolf B, Schudt C, Hermann T (2010) The preclinical pharmacology of roflumilast – a selective, oral phosphodiesterase 4 inhibitor in development for chronic obstructive pulmonary disease. *Pulm Pharmacol Ther* 23:235–326
- Hauns B, Hermann R, Hünemeyer A, Herzog R, Hauschke D, Zech K, Bethke TD (2006) Investigation of a potential food effect on the pharmacokinetics of roflumilast, an oral, once-daily phosphodiesterase 4 inhibitor, in healthy subjects. *J Clin Pharmacol* 46:1146–1153
- Harbinson PL, MacLeod D, Hawskworth R, O’Toole S, Sullivan PJ, Heath P, Kilfeather S, Page CP, Costello J, Holgate ST, Lee T (1997) The effect of a novel orally active selective PDE4 isoenzyme inhibitor (CDP840) on allergen-induced responses in asthmatic subjects. *Eur Respir J* 10:1008–1014
- Hebenstreit GF, Fellerer K, Fichte K, Fischer G, Geyer N, Meya U, Sastre-y-Hernandez M, Schöny W, Schwartzer M, Soukop W, Trampitsch E, Varosanec S, Zawada E, Zöchling R (1989) Rolipram in major depressive disorder: results of a double-blind comparative study with imipramine. *Pharmacopsychiatry* 22:156–160
- Hermann R, Lahu G, Huennemeyer A, Knoerzer D, Bethke TD, Haverkamp W (2006a) No effect of roflumilast on cardiac repolarization in healthy subjects. *Eur Respir J* 28(Suppl 50):660s

- Hermann R, Lahu G, Hauns B, Bethke T, Zech K (2006b) Total PDE4 inhibitory activity: a concept for evaluating pharmacokinetic alterations of roflumilast and roflumilast N-oxide in special populations and drug-drug interactions (abstract). *Eur Respir J* 28:436s
- Hermann R, Nassr N, Lahu G, Péterfai E, Knoerzer D, Herzog R, Zech K, de Mey C (2007a) Steady-state pharmacokinetics of roflumilast and roflumilast N-oxide in patients with mild and moderate liver cirrhosis. *Clin Pharmacokinet* 46:403–416
- Hermann R, Siegmund W, Giessmann T, Westphal K, Weinbrenner A, Hauns B, Reutter F, Lahu G, Zech K, Bethke TD (2007b) The oral, once-daily phosphodiesterase 4 inhibitor roflumilast lacks relevant pharmacokinetic interactions with inhaled budesonide. *J Clin Pharmacol* 47:1005–1013
- Hogg JC, Timens W (2009) The pathology of chronic obstructive pulmonary disease. *Annu Rev Pathol* 4:435–459
- Hohlfeld JM, Schoenfeld K, Lavae-Mohktari M, Schaumann F, Mueller F, Bredenbroeker D, Krug N, Hermann R (2008) Roflumilast attenuates pulmonary inflammation upon segmental endotoxin challenge in healthy subjects: a randomized placebo-controlled trial. *Pulm Pharmacol Ther* 21:616–623
- Horowski R, Sastre-Y-Hernandez M (1985) Clinical effects of the neurotrophic selective cAMP phosphodiesterase inhibitor rolipram in depressed patients: global evaluation of the preliminary reports. *Curr Ther Res* 38:23–29
- Huang Z, Liu S, Zhang L, Salem M, Greig GM, Chan CC, Natsumeda Y, Noguchi K (2006) Preferential inhibition of human phosphodiesterase 4 by ibudilast. *Life Sci* 78:2663–2668
- Inoue N, Harada M (2008) Effect of ibudilast on non-specific symptoms in patients with chronic cerebral ischemia. Analysis of cerebral blood flow. *Arzneimittelforschung* 58:277–282
- Kawasaki A, Hoshino K, Osaki R, Mizushima Y, Yano S (1992) Effect of ibudilast: a novel antiasthmatic agent, on airway hypersensitivity in bronchial asthma. *J Asthma* 29:245–252
- Kelly J, Murdoch RD, Clark DJ, Zussman B, Davie C, Howland K (1999) Smoking status has no effect on the clearance of a single dose of Ariflo (SB207499) (15mg), an orally active, novel, second generation PDE4 inhibitor in healthy male volunteers. *Am J Respir Crit Care Med* 159:A807
- Kelly J, Murdoch RD, Clark DJ, Webber DM, Fuder H (2001) Warfarin pharmacodynamics unaffected by cilomilast. *Ann Pharmacother* 35:1535–1539
- Keshavarzian A, Mutlu E, Guzman P, Forsyth C, Banan A (2007) Phosphodiesterase 4 inhibitors and inflammatory bowel disease: emerging therapies in inflammatory bowel disease. *Expert Opin Investig Drugs* 16:1489–1506
- Knowles RG, Tralau-Stewart C, Dawson J et al (2009) In vitro characterization of GSK256066, an exceptionally high affinity and selective inhibitor of PDE4 suitable for topical administration. *Am J Respir Crit Care Med* 179:A4581
- Krause W, Kühne G (1993) Biotransformation of the antidepressant D, L-rolipram. II. Metabolite patterns in man, rat, rabbit, rhesus and cynomolgus monkey. *Xenobiotica* 23:1277–1288
- Krause W, Kühne G, Matthes H (1989) Pharmacokinetics of the antidepressant rolipram in healthy volunteers. *Xenobiotica* 19:683–692
- Krause W, Kühne G, Sauerbrey N (1990) Pharmacokinetics of (+)-rolipram and (-)-rolipram in healthy volunteers. *Eur J Clin Pharmacol* 38:71–75
- Kuss H, Hoefgen N, Johanssen S, Kronbach T, Rundfeldt C (2003) In vivo efficacy in airway disease models of N-(3, 5-dichloro-pyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide (AWD12–281), a selective PDE4 inhibitor for inhaled administration. *J Pharmacol Exp Ther* 307:373–385
- Laazar AL, Mistry S, Barrett C, Lulic-Burns Z (2010) A four-week randomized study of the safety and tolerability of the inhaled PDE4 inhibitor GSK256066 in COPD. *Am J Respir Crit Care Med* 181:A4444 (abstract)
- Lahu G, Huennemeyer A, von Richter O, Hermann R, Herzog R, McCracken N, Zech K (2008) Effect of single and repeated doses of ketoconazole on the pharmacokinetics of roflumilast and roflumilast N-oxide. *J Clin Pharmacol* 48:1339–1349

- Lahu G, Huennemeyer A, Herzog R, McCracken N, Hermann R, Elmlinger M, Zech K (2009) Effect of repeated dose of erythromycin on the pharmacokinetics of roflumilast and roflumilast N-oxide. *Int J Clin Pharmacol Ther* 47:236–245
- Lahu G, Hünemeyer A, Diletti E et al (2010) Population pharmacokinetic modelling of roflumilast and roflumilast N-oxide by total phosphodiesterase 4 inhibitory activity and development of a population pharmacodynamic-adverse event model. *Clin Pharmacokinet* 49:589–606
- Lahu G, Nassr N, Herzog R, Elmlinger M, Ruth P, Hinder M, Huennemeyer A (2011) Effect of steady-state enoxacin on single-dose pharmacokinetics of roflumilast and roflumilast N-oxide. *J Clin Pharm* 15:586–593
- Lim S, Tomita K, Caramori G, Jatakanon A, Oliver B, Keller A, Adcock I, Chung KF, Barnes PJ (2001) Low-dose theophylline reduces eosinophilic inflammation but not exhaled nitric oxide in mild asthma. *Am J Respir Crit Care Med* 164:273–276
- Laux G, Becker T, Kühne G, Lesch KP, Riederer P, Beckmann H (1988) Clinical and biochemical effects of the selective phosphodiesterase inhibitor rolipram in depressed inpatients controlled by determination of plasma levels. *Pharmacopsychiatry* 21:378–379
- Maeda S, Ishida R, Komuro M, Ohmuro S, Hashimoto H, Hotta M, Uchida H (1989) Studies of KC-404 (3-Isobutyl-2-isopropylpyrazolo-[1,5-a]pyridine) pharmacokinetics in humans (Report 2). *Clin Rep (Kiso to Rinsho)* 23:183–189 (in Japanese)
- Mallikaarjun S, Shoaf S, Zhang P, Mitsui K, Molino NA (2006) Pharmacokinetics (PK) and pharmacodynamics (PD) of tetomilast in stable COPD subjects. *Eur Respir J* 661s, P3838 (abstract)
- Martina SD, Ismail MS, Vesta KS (2006) Cilomilast: orally active selective phosphodiesterase-4 inhibitor for treatment of chronic obstructive pulmonary disease. *Ann Pharmacother* 40:1822–1828
- McCann FE, Palfreeman AC, Andrews M, Perocheau DP, Inglis JJ, Schafer P, Feldman M, Willaims RO, Brennan FM (2010) Apremilast, a novel PDE4 inhibitor, inhibits spontaneous production of tumor necrosis factor- α from human rheumatoid synovial cells and ameliorates experimental arthritis. *Arthritis Res Ther* 12:R107
- McCracken N, Lahu G, Bethke TD (2011) Lack of pharmacokinetic and pharmacodynamic interactions of roflumilast with (R, S)-Warfarin in healthy adult subjects *J Clin Pharmacol* (in press)
- Murdoch R, Cowley H, Webber D, Tayloer H (1998) The addition of SB207499 (Ariflo), a novel & selective PDE4 inhibitor to oral prednisolone is safe & well tolerated (from study). *Eur Respir J* 12(Suppl 28):362S
- Murdoch RD, Cowley H, Kelly J, Higgins R, Webber D (2002) Cilomilast (Ariflo) does not potentiate the cardiovascular effects of inhaled salbutamol. *Pulm Pharmacol Ther* 15:521–527
- Murdoch RD, Zussman B, Schofield JP, Webber DM (2004) Lack of pharmacokinetic interactions between cilomilast and theophylline or smoking in healthy volunteers. *J Clin Pharmacol* 44: 1046–1053
- Nassr N, Lahu G, von Richter O, Reutter F, Knoerzer D, Zech K, Erb KA, Schug B, Blume H, Hermann R (2007a) Lack of a pharmacokinetic interaction between steady-state roflumilast and single-dose midazolam in healthy subjects. *Br J Clin Pharmacol* 63:365–370
- Nassr N, Lahu G, Hünemeyer A, von Richter O, Knoerzer D, Reutter F, Zech K, Hermann R (2007b) Magnesium hydroxide/aluminium hydroxide-containing antacid does not affect the pharmacokinetics of the targeted phosphodiesterase 4 inhibitor roflumilast. *J Clin Pharmacol* 47:660–666
- Nassr N, Huennemeyer A, Herzog R, von Richter O, Hermann R, Koch M, Duffy K, Zech K, Lahu G (2009) Effects of rifampicin on the pharmacokinetics of roflumilast and roflumilast N-oxide in healthy subjects. *Br J Clin Pharmacol* 68:580–587
- Nazarian R, Weinberg JM (2009) AN-2728, a PDE4 inhibitor for the potential topical treatment of psoriasis and atopic dermatitis. *Curr Opin Investig Drugs* 10:1236–1242
- Ohta K, Fukuchi Y, Grouse L, Mizutani R, Rabe KF, Rennard SI, Zhong NS (2004) A prospective clinical study of theophylline safety in 3810 elderly with asthma or COPD. *Respir Med* 98: 1016–1024

- Ohtomo H (1995) Effect of ibudilast (Ketas) in preventing the recurrence of cerebral infarction. *Yakuri to Rinsho (Jpn Pharmacol Ther)* 23:139–157
- Ohtsu H, Fujimoto Y, Yamauchi K, Tamura G, Takishima T (1989) Pharmacological effects of KC-404 on leukotriene release from human leukocytes induced by ionophore A23187. *Int Arch Allergy Appl Immunol* 89:306–310
- Pagès L, Gavaldà A, Lehner MD (2009) PDE4 inhibitors: a review of current developments (2005–2009). *Expert Opin Ther Pat* 19:1501–1519
- Peter D, Jin SL, Conti M, Hatzelmann A, Zitt C (2007) Differential expression and function of phosphodiesterase 4 (PDE4) subtypes in human primary CD4+ T-cells: predominant role of PDE4D. *J Immunol* 178:4820–4831
- Press NJ, Banner KH (2009) PDE4 inhibitors – a review of the current field. *Prog Med Chem* 47: 37–74
- Rabe KF, Bateman ED, O'Donnell D, Witte S, Bredenbrocker D, Bethke TD (2005) Roflumilast – an oral anti-inflammatory treatment for chronic obstructive pulmonary disease: a randomised controlled trial. *Lancet* 366:563–571
- Rennard SI, Schachter N, Streck M, Rickard K, Amit O (2006) Cilomilast for COPD: results of a 6-month, placebo-controlled study of a potent, selective inhibitor of phosphodiesterase 4. *Chest* 129:56–66
- Rennard S, Knobil K, Rabe KF, Morris A, Schachter N, Locantore N, Canonica WG, Zhu Y, Barnhart F (2008) The efficacy and safety of cilomilast in COPD. *Drugs* 68(Suppl 2): 3–57
- Robichaud A, Stamatou PB, Jin SL, Lachance N, MacDonald D, Laliberté F, Liu S, Huang Z, Conti M, Chan CC (2002) Deletion of phosphodiesterase 4D in mice shortens alpha(2)-adrenoceptor-mediated anesthesia, a behavioral correlate of emesis. *J Clin Invest* 110:1045–1052
- Rolan P, Gibbons JA, He L, Chang E, Jones D, Gross MI, Bahr Davidson J, Sanftner LM, Johnson KW (2008) Ibudilast in healthy volunteers: safety, tolerability and pharmacokinetics with single and multiple doses. *Br J Clin Pharmacol* 66:792–801
- Rolan P, Hutchinson MR, Johnson KW (2009) Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. *Expert Opin Pharmacother* 10:2897–2904
- Rollins BM, Burn M, Coakley RD, Chambers LA, Hirsh AJ, Clunes MT, Lethem MI, Donaldson SH, Tarran R (2008) A2B adenosine receptors regulate the mucus clearance component of the lung's innate defense system. *Am J Respir Cell Mol Biol* 39:190–197
- Sanftner LM, Gibbons JA, Gross MI, Suzuki BM, Gaeta FC, Johnson KW (2009) Cross-species comparisons of the pharmacokinetics of ibudilast. *Xenobiotica* 39:964–977
- Schafer PH, Parton A, Gandhi AK, Capone L, Adams M, Wu L, Bartlett JB, Loveland MA, Gilhar A, Cheung YF, Baillie GS, Houslay MD, Man HW, Muller GW, Stirling DI (2010) Apremilast, a cAMP phosphodiesterase-4 inhibitor, demonstrates anti-inflammatory activity in vitro and in a model of psoriasis. *Br J Pharmacol* 159:842–855
- Scott AIF, Perini AF, Shering PA, Whalley LJ (1991) In-patient major depression: is rolipram as effective as amitriptyline? *Eur J Clin Pharmacol* 40:127–129
- Shinohara Y, Kusunoki T, Nakashima M (2002) Clinical efficacy of ibudilast on vertigo and dizziness observed in patients with cerebral infarction in the chronic stage – a double-blind, randomized, placebo-controlled trial with a run-in period. *Shinkeitiryogaku (Neurol Ther)* 19:177–187
- Singh D, Petavy F, Macdonald AJ, Lazaar AL, O'Connor BJ (2010) The inhaled phosphodiesterase 4 inhibitor GSK256066 reduces allergen challenge responses in asthma. *Respir Res* 11:26
- Spina D (2008) PDE4 inhibitors: current status. *Br J Pharmacol* 155:308–315
- Stawiski MA, Rusin LJ, Burns TL, Weinstein GD, Vorhees JJ (1979) Ro 20-1724: an agent that significantly improves psoriatic lesions in double-blind clinical trials. *J Invest Dermatol* 73: 261–263
- To Y, Ito K, Kizawa Y, Failla M, Ito M, Kusama T, Elliott WM, Hogg JC, Adcock IM, Barnes PJ (2010) Targeting phosphoinositide-3-kinase delta with theophylline reverses corticosteroid insensitivity in COPD. *Am J Respir Crit Care Med* 182:897–904

- Uchida O, Ohmuro S, Hashimoto H, Irikura T (1985) Pharmacokinetics and metabolism of 3-isobutyl-2-isopropylpyrazolo [1, 5-a]pyridine (KC-404) in humans. *Clin Rep (Kiso to Rinsho)* 19:42–50
- Vakkalanka SKVS, Joshi VS, Gopalan B, Gharat LA, Husain R, Khairatkar-Joshi N, Karunakaran U, Page C (2005) GRC 3886 – A novel selective PDE4 inhibitor with reduced emetic side effects. World Congress of Inflammation, Sydney 2005 (abstract)
- Vestbo J, Prescott E, Almdal T, Dahl M, Nordestgaard BG, Andersen T, Sørensen TI, Lange P (2006) Body mass, fat-free body mass, and prognosis in patients with chronic obstructive pulmonary disease from a random population sample: findings from the Copenhagen City Heart Study. *Am J Respir Crit Care Med* 173:79–83
- Vestbo J, Tan L, Atkinson G, Ward J, UK-500, 001 Global Study Team (2009) A controlled trial of 6-weeks' treatment with a novel inhaled phosphodiesterase type-4 inhibitor in COPD. *Eur Respir J* 33:1039–1044
- von Richter O, Lahu G, Huenemeyer A, Herzog R, Zech K, Hermann R (2007) Effect of flvoxamine on the pharmacokinetics of roflumilast and roflumilast N-oxide. *Clin Pharmacokinet* 46:613–622
- Woodrow MD, Ballantine SP, Barker MD, Clarke BJ, Dawson J, Dean TW, Delves CJ, Evans B, Gough SL, Guntrip SB, Holman S, Holmes DS, Kranz M, Lindvall MK, Lucas FS, Neu M, Ranshaw LE, Solanke YE, Somers DO, Ward P, Wiseman JO (2009) Quinolines as a novel structural class of potent and selective PDE4 inhibitors. Optimisation for inhaled administration. *Bioorg Med Chem Lett* 19:5261–5265
- Zeller E, Stief HJ, Pflug B, Sastre-y-Hernández M (1984) Results of a phase II study of the antidepressant effect of rolipram. *Pharmacopsychiatry* 17:188–190
- Zhang R, Maratos-Flier E, Flier JS (2009) Reduced adiposity and high-fat diet-induced adipose inflammation in mice deficient for phosphodiesterase 4B. *Endocrinology* 150:3076–3082
- Zussman B, Kelly J, Murdoch RD, Clark DJ, Davie C, Dhaliwal J (1999) The pharmacokinetics of Ariflo (SB 207499), an orally-active novel and selective PDE4 inhibitor, are similar after morning and evening dosing. *Eur Respir J* 14:331s
- Zussman B, Kelly J, Murdoch R, Clark D, Cox P, Bush B, Benincosa L (2000) Ariflo, a novel and selective oral PDE4 inhibitor, has a favourable metabolic profile with low potential for drug-drug interactions. *Am J Respir Crit Care Med* 161:A492
- Zussman BD, Benincosa LJ, Webber DM, Clark DJ, Cowley H, Kelly J, Murdoch RD, Upward J, Wyld P, Port A, Fuder H (2001a) An overview of the pharmacokinetics of cilomilast (Ariflo), a new, orally active phosphodiesterase 4 inhibitor, in healthy young and elderly volunteers. *J Clin Pharmacol* 41:950–958
- Zussman BD, Davie CC, Kelly J, Murdoch RD, Clark DJ, Schofield JP, Walls C, Birrell C, Webber D, Quinlan J, Ritchie SY, Carr A (2001b) Bioavailability of the oral selective phosphodiesterase 4 inhibitor cilomilast. *Pharmacotherapy* 21:653–660
- Zussman BD, Kelly J, Murdoch RD, Clark DJ, Schubert C, Collie H (2001c) Cilomilast: pharmacokinetic and pharmacodynamic interactions with digoxin. *Clin Ther* 23:921–931
- Zussman B, Kelly J, Rost K, Clark DJ, Ritchie SY, Bullman SMA (2002) The effect of renal impairment on the disposition of cilomilast, a novel and selective oral PDE4 inhibitor. *Am J Respir Crit Care Med* 165(Suppl):A596
- ZuWallack RL, Mahler DA, Reilly D, Church N, Emmett A, Rickard K, Knobil K (2001) Salmeterol plus theophylline combination therapy in the treatment of COPD. *Chest* 119:1661–1670

Structural Insight into the Substrate Specificity of Phosphodiesterases

Hengming Ke, Huanchen Wang, and Mengchun Ye

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Abstract Cyclic nucleotide phosphodiesterases (PDEs) share a highly conserved catalytic domain that hydrolyzes cAMP, cGMP, or both nucleotides. However, the mechanism that allows the PDE catalytic sites to specifically recognize these nucleotides and distinguish between their subtle differences is still unclear. An early model, called the “glutamine switch”, proposed that the side chain of an invariant glutamine adopts two different conformations to allow for formation of two hydrogen bonds with cAMP and cGMP, thereby differentiating these nucleotides. However, the structure of PDE4D2 in complex with cAMP shows that Gln369 forms only one hydrogen bond with the substrate. In addition, the structures of PDE10A in complex with cAMP and cGMP reveal that cAMP and cGMP bind to the active site in different orientations and have different interactions with PDE10A residues. These structures suggest that the invariant glutamine does not appear to be a key residue to differentiate between cAMP and cGMP, although it is important for substrate binding. The structure-based sequence alignment shows that most of the active site residues change across PDE families. These residues may not only contribute differently to the substrate specificity, but also generate slightly different

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shapes and sizes of the active sites in different PDE families. Therefore, the substrate specificity of PDEs is likely to be determined jointly by multiple elements at the active site, yet the detailed mechanism needs further study.

Keywords cAMP · cGMP · Crystal structure · Substrate specificity

1 Introduction

The second messengers adenosine and guanosine 3', 5'-cyclic monophosphate (cAMP and cGMP) mediate the response of cells to a wide variety of hormones and neurotransmitters and modulate many physiological processes, including cardiac and smooth muscle contraction, steroid hormone function, platelet aggregation, apoptosis, leukocyte migration, adrenal hyperplasia, inflammation, axon guidance and regeneration, memory, and circadian regulation (Houslay 1998; Antoni 2000; Zaccolo and Movsesian 2007; Piper et al. 2007; O'Neill et al. 2008; Horvath and Stratakis 2008; Hannila and Filbin 2008). The signaling of cAMP and cGMP in vivo involves three types of enzymes: cyclases, cyclic nucleotide (cN) phosphodiesterases (PDEs), and cN-dependent protein kinases. PDEs are the sole enzymes that hydrolyze cN (Fig. 1) and thus play pivotal roles in these cN signaling pathways.

The human genome contains 21 PDE genes that are classified into 11 families and encode more than 100 isoforms of PDE proteins via alternative mRNA splicing (Bender and Beavo 2006; Omori and Kotera 2007; Conti and Beavo 2007). PDE molecules can typically be divided into a variable regulatory domain at the N-terminus and a conserved catalytic domain at the C-terminus; in some instances, additional regulatory elements are found near the C-terminus. The conserved catalytic domains show different substrate preferences. PDEs 4, 7, and 8 preferentially hydrolyze cAMP while PDEs 5, 6, and 9 are specific for cGMP. PDEs 1, 2, 3, 10, and 11 possess activities toward both nucleotides and are known as dual-specific

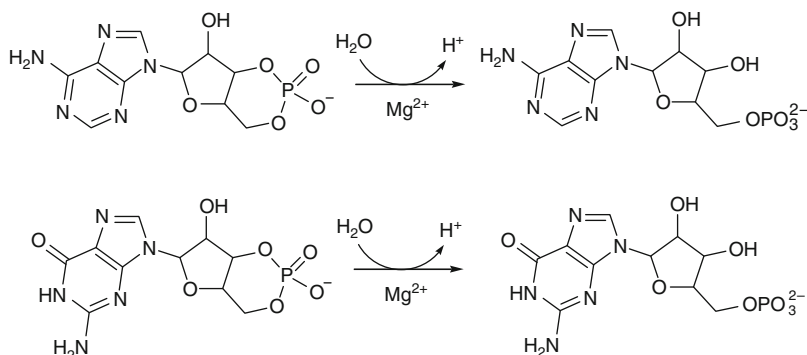


Fig. 1 Hydrolysis of cAMP and cGMP to 5'-AMP and 5'-GMP by PDEs

PDEs (Conti and Beavo 2007; Lugnier 2006). This chapter reviews the progress on the understanding of structural features that contribute to determining the substrate specificity of these enzymes.

2 The Active Site of PDEs

The first crystal structure of the PDE4B catalytic domain (Xu et al. 2000) and the later structure of the PDE4D2 catalytic domain in complex with AMP (Huai et al. 2003) provide a full definition of the active sites of PDE families. The active sites of PDEs can be divided into two major subpockets: a cN-binding subpocket and a divalent metal-binding subpocket (Fig. 2). All the PDE families contain two divalent metal ions that are essential for their catalytic functions. The first metal was identified by X-ray anomalous scattering as zinc and coordinates with four invariant residues (His164, His200, Asp201, and Asp318 in PDE4D2, Table 1) and two water molecules in an octahedral configuration (Fig. 2b). The second metal also forms six co-ordinations with an aspartic acid (Asp201 in PDE4D2) and five waters in an octahedral configuration, but its chemical nature is not clear. Magnesium has been in general accepted as the second catalytic ion for all PDE families, although early reports showed that other divalent metal ions such as manganese and cobalt promote the catalytic activity as effectively as does magnesium (Hitchcock 1973). Besides, manganese was shown to support twice the catalytic activity of PDE9 and PDE8 as that supported by magnesium (Fisher et al. 1998; Huai et al. 2004; Wang et al. 2008). Moreover, the affinity of PDE5 for manganese is 30–100-fold that for magnesium, although maximum catalytic activity is similar for the two metal ions

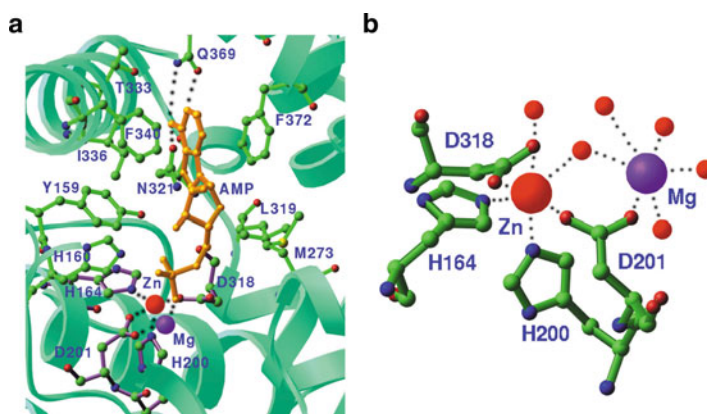


Fig. 2 Active site pocket of PDE4D2 in complex with 5'-AMP. (a) The product (5'-AMP) binding at the active site. The dotted lines represent hydrogen bonds or metal coordinations. (b) Each of two divalent metal ions forms six coordinations (*dotted lines*) in octahedral conformation. The small red balls are water molecules. The identity of the second metal has not been determined, but is depicted here as magnesium

Table 1 Alignment of residues at the active sites of PDEs

	159	160	164	200	201	204	273	318	319	321	322	329	333	336	337	340	357	368	369	372	373	376	406
Pde4D2	Y	H	H	H	D	H	M	D	L	N	P	Y	T	I	M	F	M	S	Q	F	I	I	Y
Pde7	Y	H	H	H	D	H	I	D	I	N	P	S	S	V	T	F	L	I	Q	F	M	L	W
Pde8	Y	H	H	H	D	H	M	D	V	N	P	C	A	I	S	Y	V	S	Q	F	I	F	W
Pde1	Y	H	H	H	D	H	M	D	I	H	P	H	T	L	M	F	L	S	Q	F	I	I	S/N/G ^a
Pde2	Y	H	H	H	D	H	L	D	L	D	Q	T	A	I	Y	F	M	L	Q	F	M	I	W
Pde3	Y	H	H	H	D	H	L	D	I	G	P	H	T	I	V	F	F	L	Q	F	I	I	W
Pde10A2	Y	H	H	H	D	H	L	D	L	S	V	T	A	I	Y	F	M	G	Q	F	Y	V	W
Pde11	Y	H	H	H	D	H	L	D	L	A	V	S	A	V	T	F	F	L	Q	W	I	I	W
Pde5	Y	H	H	H	D	H	L	D	L	A	I	Q	A	V	A	F	M	M	Q	F	I	I	W
pde6	Y	H	H	H	D	H	L	D	L	A	I	Q	A	V	A	F	M	L	Q	F	I	V	W
Pde9A2	F	H	H	H	D	H	M	D	I	N	E	A	V	L	L	Y	F	A	Q	F	I	Y	Y

The first row of the table is the amino acid number in PDE4D2

^aS/N/G represent Ser, Asn, and Gly in PDE1A, 1B, and 1C, respectively

^bMetal binding residues

(Francis et al. 2006). Thus, it remains puzzling whether the second metal ion is magnesium or manganese in biological systems and whether different PDE families prefer different divalent metals.

The structure-based sequence alignment reveals the conservation of residues in the active site of PDE families (Table 1). In addition to the four invariant metal-binding residues, three residues at the active sites are invariant across PDE families (His160, His204, and Gln369 in PDE4D2). His160 was proposed to serve as a general acid for the catalysis (Huai et al. 2003; Liu et al. 2008), while the exact role of His204 remains to be identified. In the nucleotide-binding pocket, the invariant glutamine (Gln369 in PDE4D2, Table 1) forms at least one hydrogen bond with substrates or inhibitors, and a conserved phenylalanine (tryptophan in PDE11) stacks against substrates and inhibitors (Fig. 2). These interactions are two characteristics for binding of substrates or inhibitors in all PDE families.

3 Substrate Specificity

After the first report of the isolation of the cAMP-specific PDE from beef heart (Butcher and Sutherland 1962), various isoforms of PDEs were discovered to have different capabilities for hydrolyzing cAMP and cGMP (Beavo et al. 1970; Monn and Christiansen 1971). For over 48 years, the essential characteristics of the mechanism whereby the conserved catalytic domains of PDE families selectively recognize the subtle differences between cAMP and cGMP have remained elusive. In kinetic theory, the substrate specificity is quantitatively defined by the ratio of the specific constants of one substrate versus another, specifically by $(k_{\text{cat}}/K_{\text{M}})^{\text{cAMP}}$ over $(k_{\text{cat}}/K_{\text{M}})^{\text{cGMP}}$ for PDEs. However, since the pure PDEs, and thus the k_{cat} values, were not easily obtained in early times, the apparent equilibrium constant K_{M} has been conveniently used to represent the substrate specificity of PDEs (Mehats et al. 2002). In recent years, due to great progress on the expression and purification of high quality recombinant PDEs, the k_{cat} values and the specific constants can now be measured, and thus the substrate specificity can be fully defined. As shown in Table 2, the K_{M} is indeed dominant in determination of the substrate specificity. However, caution should be taken for evaluation of the parameters, respectively, obtained from the catalytic domain and full-length enzymes. Although several PDE families show similar kinetic parameters for the catalytic domains and the full-length enzymes, the allosteric modes of certain PDE families may have different K_{M} and k_{cat} of these enzymes. In addition, the potential dimerization of some highly purified PDEs may offer the possibility of alterations in k_{cat} , relative to a monomer. For example, the PDE4 model suggests that a long-form dimer may have an activity that is half that of the dimer due to capping of one of the active site in one partner by a regulatory portion of the other partner (Burgin et al. 2010).

Table 2 Substrate specificity of the PDE catalytic domains^a

Enzyme	cAMP			cGMP			Specificity (k_{cat}/K_M) ^{cAMP} / (k_{cat}/K_M) ^{cGMP}
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}M^{-1}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}M^{-1}$)	
PDE4D2(1-507)	1.5 ± 0.2	3.9 ± 0.3	2.7 ± 0.4 × 10 ³	1.0 ± 0.1 × 10 ³	5.2 ± 0.8	5.3 ± 0.8	509
PDE7A1(130-482)	0.2 ± 0.03	1.6 ± 0.2	7.9 ± 0.1 × 10 ³	3.9 ± 0.7 × 10 ³	6.8 ± 1.3	1.8 ± 0.5	4,400
PDE8A1(480-820)	1.8 ± 0.1	4.0 ± 0.1	2.2 ± 0.1 × 10 ³	1.6 ± 0.1 × 10 ³	1.6 ± 0.2	1.0 ± 0.1	2,200
PDE5A1(535-875)	3.0 ± 0.8 × 10 ³	2.1 ± 0.8	0.69 ± 0.07	5.1 ± 1.3	1.3 ± 0.3	0.27 ± 0.08 × 10 ³	1/391
PDE9A2(181-506)	0.2 ± 0.02 × 10 ³	0.05 ± 0.01	0.28 ± 0.05	0.14 ± 0.02	1.0 ± 0.2	7.0 ± 0.07 × 10 ³	1/25,000
PDE10A2(448-789)	0.056 ± 0.005	0.33 ± 0.02	5.9 ± 0.6 × 10 ³	4.4 ± 0.3	1.2 ± 0.1	0.27 ± 0.01 × 10 ³	22
PDE4D	0.5–4.0						
PDE5A				2.0			
PDE9A				0.07–0.39			

^aPDE4D2 is the full-length protein. References are as follows: PDE4D2 and PDE7A1 (Wang et al. 2005), PDE8A1 (Wang et al. 2008; Yan et al. 2008), PDE5A1 (Wang et al. 2006); PDE9A2 (Huai et al. 2004), PDE10A2 (Wang et al. 2007a); full length PDE4D (Bolger et al. 1997; Mehats et al. 2002); PDE5A (Turko et al. 1998); PDE9A (Soderling et al. 1998; Fisher et al. 1998)

4 An Early Proposal for the Substrate Specificity: “Glutamine Switch”

In the analysis of the first X-ray crystal structure of a PDE4B catalytic domain, Xu et al. (2000) proposed that the orientation of the side chain of the invariant glutamine in PDEs might play a strict role to select substrates cAMP and cGMP. In 2004, Zhang et al. advanced this concept and called it the “glutamine switch”. This hypothesis was based on the crystal structures of PDE4 and PDE5 in complex with their products 5'-AMP and 5'-GMP (Zhang et al. 2004). The crystal structures showed that the side chain of the invariant glutamine is fixed in opposite orientations in the nucleoside-binding pockets of PDE4 and PDE5 and forms two hydrogen bonds with 5'-AMP and 5'-GMP, respectively (Fig. 3). These workers assumed that the binding of the products simulates binding of the guanine or adenine portion of the substrates. A 180° rotation of the glutamine side chain in either enzyme would allow for formation of only one hydrogen bond with the less preferred substrate. For the PDE families with a dual-specificity of the substrates, according to the “glutamine switch” hypothesis, the invariant glutamine side chain would be free to rotate and therefore form two hydrogen bonds when either cAMP or cGMP binds (Fig. 3). Thus, the difference in the orientation of the glutamine side chain would make a difference of one hydrogen bond and account for the substrate preference of the enzymes. The “glutamine switch” mechanism was supported by the structures of dual cAMP/cGMP-specific PDE1B (Zhang et al. 2004) and PDE3B (Scapin et al. 2004), in which the invariant glutamine was not bonded with other protein residues and was therefore free to rotate.

5 Evidence Against the “Glutamine Switch” Mechanism

Several lines of evidence suggest that the glutamine switch mechanism, while being conceptually inviting, may be “too good to be true”. The structure of PDE2A3, which has similar affinity for cAMP and cGMP and hydrolyzes both substrates equally well, shows that the side chain of the invariant glutamine (Gln859) is fixed by a hydrogen bond with Tyr827 in the unliganded state of the enzyme (Iffland et al. 2005). In addition, the recently published structure of PDE10A2 showed Gln726 is locked in place by two hydrogen bonds; one bond involves Tyr693 and the other occurs through a water that is bound to Tyr730 and Trp762 (Wang et al. 2007a). Thus, in order for the invariant glutamine in either PDE2 or PDE10 to switch orientation, these pre-existing hydrogen bonds would have to be broken, a process that would be energetically costly. On the contrary, the Q817A mutation of PDE5 reduced K_M for cGMP by 60-fold, but did not significantly change K_M for cAMP (Zoraghi et al. 2006). Since the Gln817 side chain should have been free to rotate in the Q759A mutant, it would have been predicted to improve affinity for cAMP. Furthermore, mutation of the amino acid (Gln759) that tethers the side chain of the

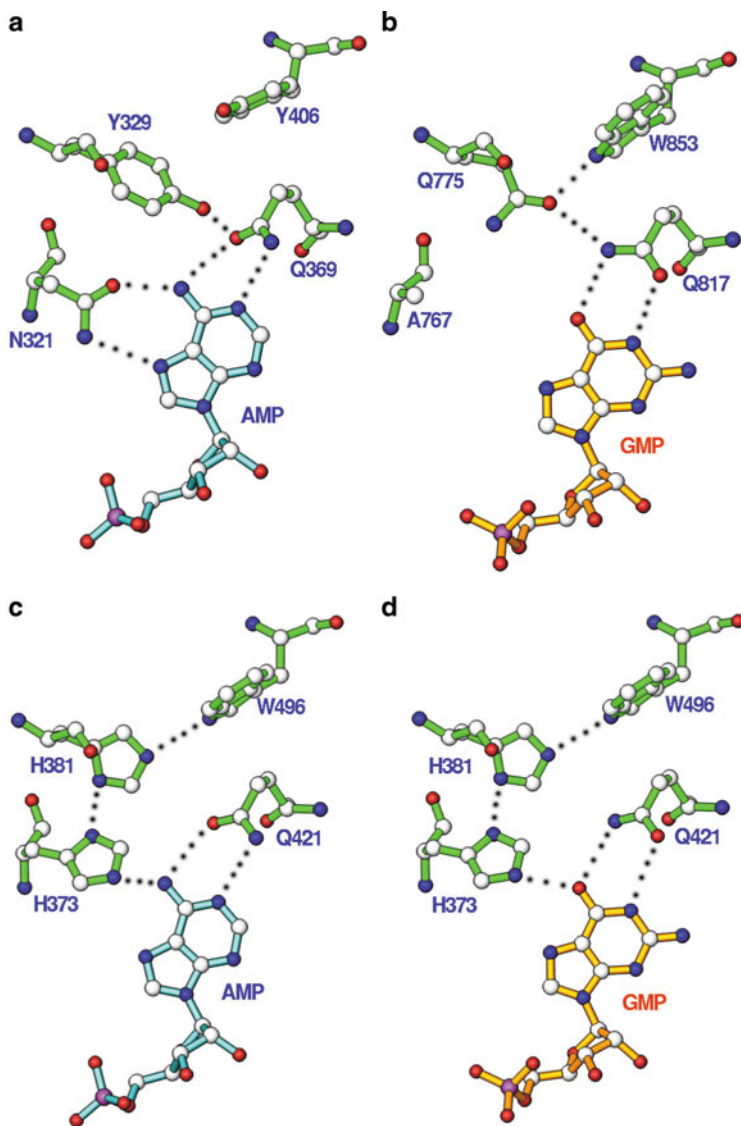


Fig. 3 The proposed mechanism of the “glutamine switch” (Zhang et al. 2004). (a) Gln369 forms two hydrogen bonds (*dotted lines*) with the product 5'-AMP in the cAMP-specific PDE4D2 structure. (b) Gln817 of the cGMP-specific PDE5A1 is in an orientation opposite to that of Gln369 and forms two hydrogen bonds with 5'-GMP. (c) and (d) Gln421 in the dual-specific PDE1B switches its side chain conformation to form two hydrogen bonds with 5'-AMP or 5'-GMP

invariant Gln817 decreased affinity for cGMP, but did not improve affinity for cAMP (Zoraghi et al. 2006). These combined results suggest that the invariant glutamine in PDEs is important for the substrate binding, but that the orientation of its side chain may not be a key factor for the differentiation of cAMP and cGMP.

6 Binding of the Products Does Not Simulate Binding of the Substrates

The glutamine switch mechanism is based on the assumption that the products simulate the binding of the substrates because they share the nucleoside portions. However, since products of enzymatic reactions serve as leaving groups and often have much lower affinity than substrates, significant differences in binding of substrates and products are expected and therefore the suitability of the model is in question. To this purpose, the structure of a PDE4D2 mutant in complex with cAMP was determined at high resolution (Wang et al. 2007b). The superposition of PDE4D2-cAMP over PDE4D2-AMP (Huai et al. 2003) yielded the small RMSD of 0.26 Å, indicating overall similarity of the protein structures. In addition, cAMP and 5'-AMP have the same *anti* configuration and interact with a similar set of amino acids (Fig. 4). However, the hydrogen bonding patterns of cAMP and 5'-AMP in the structures of PDE4D2-cAMP and PDE4D2-AMP are very different (Fig. 4a). First, a phosphate oxygen of 5'-AMP bridges the two divalent metal ions, but cAMP does not directly contact the metal ions. Second, the side chain of Asn321 in the

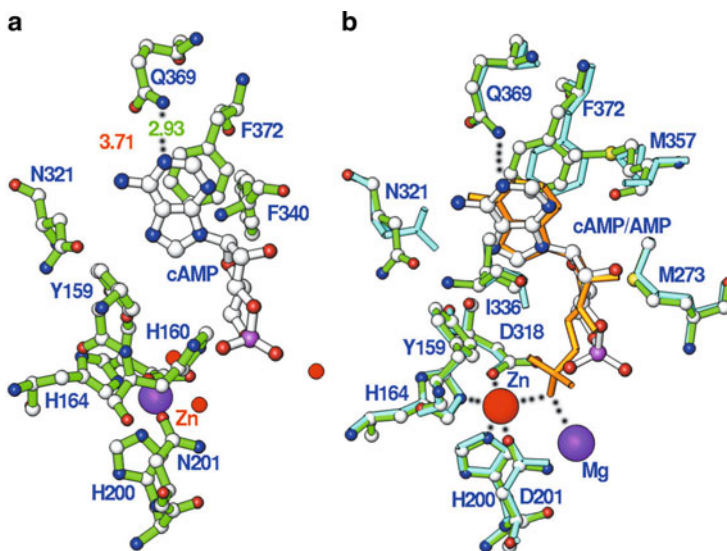


Fig. 4 Binding of substrate cAMP and product 5'-AMP in the PDE4D2 structures. (a) Binding of cAMP to the pocket of PDE4D2. Gln369 forms only one hydrogen bond (dotted line) with cAMP. (b) Superposition of cAMP (white bonds) over 5'-AMP (golden bonds). The green colored bonds represent residues from the PDE4D2-cAMP structures, while cyan is for residues of PDE4D2-AMP. The side chain conformation of Asn321 is different in the two structures. Phe372 shows some positional changes while other residues have no significant difference

PDE4D2-AMP complex changes its conformation in the unliganded state to form two hydrogen bonds with N₆ and N7 of 5'-AMP. In comparison, Asn321 in the PDE4D2-cAMP complex when compared with the unliganded state retains its conformation and does not form hydrogen bonds with cAMP. Finally, two hydrogen bonds are formed between N1 and N₆ of 5'-AMP and Nε2 and Oε1 of Gln369 in the PDE4D2-AMP complex. In contrast, Gln369 in the PDE4D2-cAMP structure forms only one hydrogen bond with N1 of cAMP, and the distance of 3.7 Å between Oε1 of Gln369 and N₆ of cAMP indicates their weak van der Waals' interaction (Fig. 4a). The observation of only one hydrogen bond between cAMP and the invariant glutamine in the PDE4D2-cAMP structure provides direct evidence against the mechanism of "glutamine switch", in which two hydrogen bonds were assumed (Zhang et al. 2004).

Further support for the argument that the 5'-nucleotide products are not good models for substrates comes from the structures of PDE10A (Wang et al. 2007a). These structures showed that both substrates cAMP and cGMP bound to PDE10A with the *syn* configuration, but the products (5'-AMP and 5'-GMP) are in the *anti* configuration (Fig. 5). These different conformations lead to different interactions in terms of hydrogen bonding and van der Waal's contacts. In summary, the different conformations between the substrates and products in the PDE10 structures and the different hydrogen bonding pattern in PDE4 strongly support the conclusion that the products of cN hydrolysis are not reasonable models for describing the details of substrate binding.

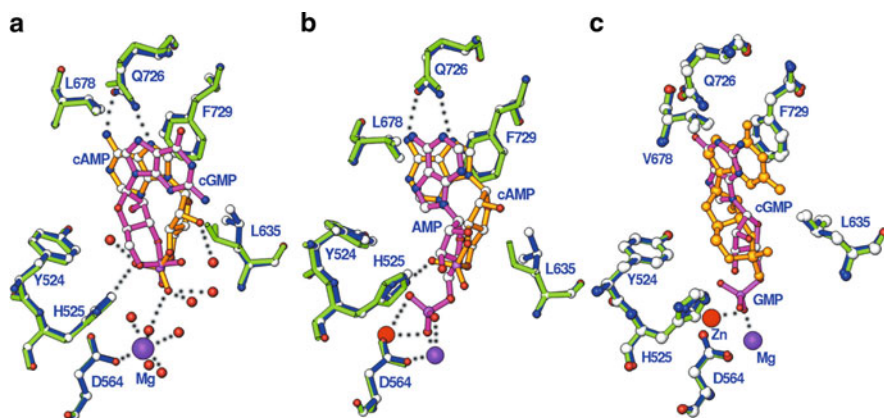


Fig. 5 Comparison of binding of substrates and products in the PDE10 structures. (a) cAMP (golden bonds) has an orientation different from cGMP (pink bonds). The colors blue and green represent residues from the cAMP and cGMP structures, respectively. (b) Superposition of PDE10-cAMP (blue and golden bonds) over PDE10-AMP (green and pink bonds). Dotted lines represent hydrogen bonds or coordination with the metal ions. (c) Superposition of PDE10-cGMP (blue and golden bonds) over PDE10-GMP (green and pink bonds)

7 Equilibrium of *Syn/Anti* Configuration of cAMP and cGMP

It has long been known that cAMP and cGMP exist in equilibrium of *syn* and *anti* configurations in solution, with *syn/anti* ratios of 30:70 and 95:5, respectively (Yathindra and Sunderalingam 1974), in contrast to the predominant *anti* configuration of the products, 5'-AMP and 5'-GMP (Lee et al. 1975). The early studies of PDE catalytic function using cN analogs rationalized that the preferred configuration for the cN substrates was: *syn* for cAMP and cGMP in PDE1 and PDE2, *anti* for cAMP in PDE3 and PDE4, and *anti* for cGMP in PDE3 and PDE5 (Butt et al. 1995a, b). The crystal structures show only the *syn* configuration of cAMP and cGMP bound to the active site of PDE10A2 (Wang et al. 2007a), *anti*-cAMP in PDE4D (Wang et al. 2007b), and *anti*-cGMP in PDE9A (Liu et al. 2008).

Since the PDE10A structures showed *syn* configuration for cAMP and cGMP, and *anti* for AMP and GMP (Fig. 5), it is a question whether PDE10 requires an additional step in the catalytic process to perform the *syn* to *anti* conversion before the product is released. We hypothesize that the *syn* product leaves the active site immediately after hydrolysis and that the *syn* to *anti* conversion is an automatic process of equilibrium in solution. This is supported by the observation that only one configuration of the substrates is identified in the PDE10 structures, and also by the fact that the products (5'-AMP and 5'-GMP) in the *anti* configuration have very poor affinity for the enzymes, typically in the millimolar range (our unpublished data). The occupancy of the *anti* 5'-AMP and 5'-GMP at the active sites of the PDEs in the X-ray crystal structures may reflect artifactual binding of the products at very high concentration.

8 The Substrate Specificity Is Determined Jointly by Multiple Elements

The different orientations and interactions of cAMP and cGMP in the PDE10 structures suggest that the substrate specificity is determined by the combined influence of multiple elements in the binding pocket. The structure-based sequence alignment of the nucleoside-binding pocket, that we tentatively termed “substrate-specificity pocket” or S-pocket (Wang et al. 2007a), shows significant variation of amino acids across PDE families (Table 1, Fig. 6). Apparently, the amino acid variation not only changes the chemical nature of this pocket among PDE families, but also renders the different shape and size of the binding pockets. To match the chemical nature and shape of the pocket, substrates may bind to individual PDE families in either the same or different orientations and configurations with different affinities. Although the differences between the binding pockets of the PDE families may not be dramatic (Fig. 6), they may be sufficient for recognition of different substrates. Most likely, the dual-specific PDE families will take both substrates with the same configuration but different interactions, as shown in the case of PDE10 (Wang et al. 2007a). In the cAMP- or cGMP-specific PDE families,

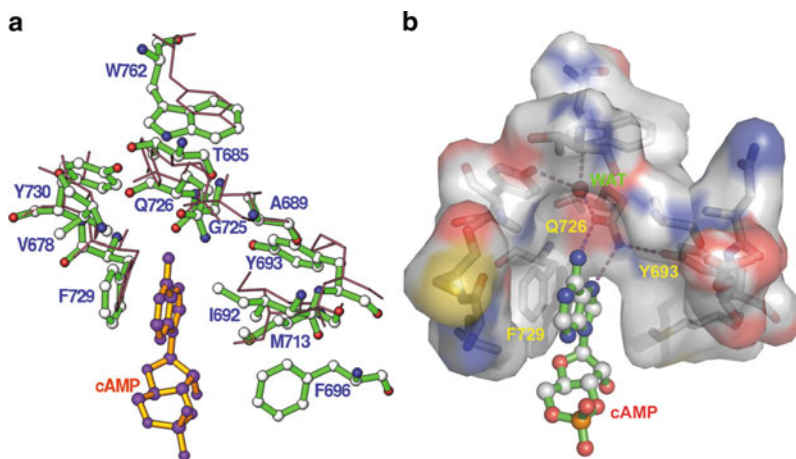


Fig. 6 The putative substrate-specificity pocket (S-pocket). (a) The PDE10A2 residues (green bonds) are superimposed over the PDE4D2 residues (thinner salmon sticks). (b) Surface presentation of the S-pocket in PDE10

one substrate would not effectively bind to the pocket, due to the poor apparent affinity constants (K_M) in the millimolar range (Table 2).

In summary, the structural and biochemical studies support the interpretation that the substrate specificity of PDEs is jointly determined by multiple elements in the PDE catalytic S-pocket and that no individual residues play a dominant role. Moreover, the structural data suggests that amino acids in the S-pocket may contribute importantly and distinctly to the substrate specificity in different PDE families, yet their quantitative roles in this process need further study.

References

- Antoni F (2000) Molecular diversity of cyclic AMP signaling. *Front Neuroendocrinol* 21:103–132
- Beavo JA, Hardman JG, Sutherland EW (1970) Hydrolysis of cyclic guanosine and adenosine 3', 5'-monophosphates by rat and bovine tissues. *J Biol Chem* 245:5649–5655
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Bolger GB, Erdogan S, Jones RE, Loughney K, Scotland G, Hoffmann R, Wilkinson I, Farrell C, Houslay MD (1997) Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene. *Biochem J* 328:539–548
- Burgin AB, Magnusson OT, Singh J, Witte P, Staker BL, Bjornsson JM, Thorsteinsdottir M, Hrafnisdottir S, Hagen T, Kiselyov AS, Stewart LJ, Gurney ME (2010) Design of phosphodiesterase 4D (PDE4D) allosteric modulators for enhancing cognition with improved safety. *Nat Biotechnol* 28:63–70
- Butcher RW, Sutherland EW (1962) Adenosine 3', 5'-phosphate in biological materials. I. purification and properties of cyclic 3'5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3', 5'-phosphate in human urine. *J Biol Chem* 237:1244–1250

- Butt E, Beltman J, Becker DE, Jensen GS, Rybalkin SD, Jastorff B, Beavo JA (1995a) Characterization of cyclic nucleotide phosphodiesterases with cyclic GMP analogs: topology of the catalytic domains. *Mol Pharmacol* 47:330–339
- Butt E, Beltman J, Becker DE, Jensen GS, Rybalkin SD, Jastorff B, Beavo JA (1995b) Characterization of cyclic nucleotide phosphodiesterases with cyclic AMP analogs: topology of the catalytic sites and comparison with other cyclic AMP-binding proteins. *Mol Pharmacol* 47:340–347
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- Fisher DA, Smith JF, Pillar JS, St Denis SH, Cheng JB (1998) Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. *J Biol Chem* 273:15559–15564
- Francis SH, Zoraghi R, Kotera J, Ke H, Bessay EP, Blouni MA, Corbin JD (2006) Phosphodiesterase 5: Molecular characteristics relating to structure, function, and regulation. In: Beavo JA, Francis SH, Houslay MS (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, FL, pp 131–164
- Hannila SS, Filbin MT (2008) The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. *Exp Neurol* 209:321–332
- Hitchcock M (1973) Adenosine 3', 5'-cyclic monophosphate phosphodiesterase in guinea-pig lung-properties and effect of adrenergic drugs. *Biochem Pharmacol* 22:959–969
- Horvath A, Stratakis CA (2008) Unraveling the molecular basis of micronodular adrenal hyperplasia. *Curr Opin Endocrinol Diabetes Obes* 15:227–233
- Houslay MD (1998) Adaptation in cyclic AMP signaling processes: a central role for cyclic AMP phosphodiesterases. *Cell Develop Biol* 9:161–167
- Huai Q, Colicelli J, Ke H (2003) The crystal structure of AMP-bound PDE4 suggests a mechanism for phosphodiesterase catalysis †, ‡. *Biochemistry* 42:13220–13226
- Huai Q, Wang H, Zhang W, Colman R, Robinson H, Ke H (2004) Crystal structure of phosphodiesterase 9 shows orientation variation of inhibitor 3-isobutyl-1-methylxanthine binding. *Proc Natl Acad Sci USA* 101:9624–9629
- Iffland A, Kohls D, Low S, Luan J, Zhang Y, Kothe M, Cao Q, Kamath AV, Ding YH, Ellenberger T (2005) Structural determinants for inhibitor specificity and selectivity in PDE2A using the wheat germ *in vitro* translation system. *Biochemistry* 44:8312–8325
- Lee CH, Evans FE, Sarma RH (1975) Interaction between glycosidic torsion, sugar pucker, and backbone conformation in 5'-b-nucleotides. *J Biol Chem* 250:1290–1296
- Liu S, Mansour MN, Dillman KS, Perez JR, Danley DE, Aeed PA, Simons SP, Lemotte PK, Menniti FS (2008) Structural basis for the catalytic mechanism of human phosphodiesterase 9. *Proc Natl Acad Sci USA* 105:13309–13314
- Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 109:366–398
- Mehats C, Andersen CB, Filopanti M, Jin SL, Conti M (2002) Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. *Trends Endocrinol Metab* 13:29–35
- Monn E, Christiansen RO (1971) Adenosine 3', 5'-monophosphate phosphodiesterase: multiple molecular forms. *Science* 173:540–542
- Omori K, Kotera J (2007) Overview of PDEs and their regulation. *Circ Res* 100:309–327
- O'Neill JS, Maywood ES, Chesham JE, Takahashi JS, Hastings MH (2008) cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. *Science* 320:949–953
- Piper M, van Horck F, Holt C (2007) The role of cyclic nucleotides in axon guidance. *Adv Exp Med Biol* 621:134–143
- Scapin G, Patel SB, Chung C, Varnerin JP, Edmondson SD, Mastracchio A, Parmee ER, Singh SB, Becker JW, Van der Ploeg LH, Tota MR (2004) Crystal structure of human phosphodiesterase 3B: atomic basis for substrate and inhibitor specificity. *Biochemistry* 43:6091–6100
- Soderling SH, Bayuga SJ, Beavo JA (1998) Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. *J Biol Chem* 273:15553–15558

- Turko IV, Francis SH, Corbin JD (1998) Hydropathic analysis and mutagenesis of the catalytic domain of the cGMP-binding cGMP-specific phosphodiesterase (PDE5). cGMP versus cAMP substrate selectivity. *Biochemistry* 37:4200–4205
- Wang H, Liu Y, Chen Y, Robinson H, Ke H (2005) Multiple elements jointly determine inhibitor selectivity of cyclic nucleotide phosphodiesterases 4 and 7. *J Biol Chem* 280:30949–30955
- Wang H, Liu Y, Huai Q, Cai J, Zoraghi R, Francis SH, Corbin JD, Robinson H, Xin Z, Lin G, Ke H (2006) Multiple conformations of phosphodiesterase-5: implications for enzyme function and drug development. *J Biol Chem* 281:21469–21479
- Wang H, Liu Y, Hou J, Zheng M, Robinson H, Ke H (2007a) Structural insight into substrate specificity of phosphodiesterase 10. *Proc Natl Acad Sci USA* 104:5782–5787
- Wang H, Robinson H, Ke H (2007b) The molecular basis for recognition of different substrates by phosphodiesterase families 4 and 10. *J Mol Biol* 371:302–307
- Wang H, Yan Z, Yang S, Cai J, Robinson H, Ke H (2008) Kinetic and structural studies of phosphodiesterase-8A and implication on the inhibitor selectivity. *Biochemistry* 47:12760–12768
- Xu RX, Hassell AM, Vanderwall D, Lambert MH, Holmes WD, Luther MA, Rocque WJ, Milburn MV, Zhao Y, Ke H, Nolte RT (2000) Atomic structure of PDE4: Insight into phosphodiesterase mechanism and specificity. *Science* 288:1822–1825
- Yan Z, Wang H, Cai J, Ke H (2008) Refolding and kinetic characterization of the phosphodiesterase-8A catalytic domain. *Protein Expr Purif* 64:82–88
- Yathindra N, Sunderalingam M (1974) Conformations of cyclic 3', 5'-nucleotides. Effect of the base on the Syn-Anti conformer distribution. *Biochem Biophys Res Commun* 56:119–126
- Zaccolo M, Movsesian MA (2007) cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res* 100:1569–1578
- Zhang KY, Card GL, Suzuki Y, Artis DR, Fong D, Gillette S, Hsieh D, Neiman J, West BL, Zhang C, Milburn MV, Kim SH, Schlessinger J, Bollag G (2004) A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases. *Mol Cell* 15:279–286
- Zoraghi R, Corbin JD, Francis SH (2006) Phosphodiesterase-5 Gln817 is critical for cGMP, vardenafil, or sildenafil affinity; its orientation impacts cGMP but not cAMP affinity. *J Biol Chem* 281:5553–5558

A Fission Yeast-Based Platform for Phosphodiesterase Inhibitor HTSs and Analyses of Phosphodiesterase Activity

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Abstract Fission yeast strains have been engineered so that their growth behavior reflects the activity of heterologous cyclic nucleotide phosphodiesterases (PDEs). These strains can be used in High-Throughput Screens (HTSs) for PDE inhibitors that possess “drug-like” characteristics, displaying activity in a growth stimulation assay over a 48-h period. Through three generations of development, a collection of strains expressing 10 of the 11 mammalian PDE families that is appropriate for small molecule inhibitor screening has been generated in our laboratory. Strains unable to synthesize cyclic nucleotides allow characterization of PDE activity in that the enzyme’s potency is reflected in the amount of either cAMP or cGMP that must be added to the growth medium to stimulate cell growth. In the future, this system could be used to screen cDNA libraries for biological regulators of target PDEs and for the construction of strains that co-express PDEs and associated regulatory proteins to facilitate molecular and genetic studies of their functions and, in particular, to identify whether different PDE-partner protein complexes show distinct patterns of inhibitor sensitivity.

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1 Introduction

Mammals express 11 families of cyclic nucleotide phosphodiesterases (PDEs), encoded by 21 genes that generate more than 100 distinct PDE isoforms by variations in transcriptional start sites and splicing. Each PDE family includes proteins encoded by as many as four genes (designated A through D), and are grouped, in part, according to substrate specificity: PDE4, PDE7, and PDE8 are cAMP-specific; PDE5, PDE6, and PDE9 are cGMP-specific; and PDE1, PDE2, PDE3, PDE10, and PDE11 display dual-specificity. PDE families are also grouped according to conserved domains outside of the catalytic domain and their relative sensitivity to various chemical inhibitors and other molecules such as calcium/calmodulin or cGMP (Bender and Beavo 2006; Conti and Beavo 2007; Lerner and Epstein 2006; Soderling and Beavo 2000). In addition, tissue-specific expression and subcellular localization of PDEs permit members of this superfamily, though acting on only two substrates, to influence tissue-specific biological processes or even distinct processes in a single cell through compartmentalization of cAMP signaling (Houslay 2010). Thus, inhibition of a specific PDE subpopulation in a cell may have a therapeutic benefit without altering other cAMP- or cGMP-controlled processes in the affected cell.

Most, though not all, approaches to PDE inhibitor development focus on compounds that act by binding to the catalytic site. This is true of medicinal chemistry approaches that produce and characterize analogs of nonselective PDE inhibitors, as well as rational drug design approaches that are guided by crystal structures of target PDEs (Card et al. 2004, 2005). Although these methods have led to the development of many selective PDE inhibitors, there remains a need to develop specific inhibitors for certain PDE families (PDE1, PDE6, PDE8, PDE11), as well as subtype-selective inhibitors for families encoded by multiple genes (PDE1, PDE3, PDE4, PDE6, PDE7, and PDE8).

This chapter describes a fission yeast-based platform for PDE inhibitor screens that can also be used to characterize PDEs in live cells. Differing from traditional inhibitor screens, this approach allows inexpensive screening of full-length enzymes expressed in a eukaryotic cell, following the yeast genetic philosophy of discovery: “first find something that does what you are looking for and then figure out the mechanism”. As such, this screening platform is open to the discovery of both active-site and allosteric inhibitors that are structurally unrelated to current PDE inhibitors. In addition, it can be used to characterize the PDEs under conditions that closely resemble their natural cellular environment in contrast to *in vitro* enzyme assays that are frequently carried out on biochemically stable fragments of PDEs expressed in and purified from *E. coli*. Finally, this system is amenable to conducting cDNA library screens for genes that encode biological regulators of a target PDE and genetic screens for mutations that affect PDEs or their regulators.

2 Use of Yeast to Study Mammalian Proteins

As unicellular eukaryotes, the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* serve as important model organisms for the study of biological processes that are conserved in human cells. Both yeasts have been used to clone human genes by functional complementation of a defective gene in the host yeast strain. For example, the human Cdc2 cyclin-dependent kinase gene was first cloned by its ability to suppress the temperature-sensitive growth of a *S. pombe cdc2*⁻ mutant strain (Lee and Nurse 1987). Yeast strains expressing human proteins are used to study the function of the human protein and to identify mutations that alter function, as in a study of the p53 protein expressed in *S. pombe* (Bischoff et al. 1992). Furthermore, yeast growth-based High-Throughput Screens (HTSs) have been successfully deployed to identify inhibitors of various eukaryotic proteins, including sirtuin family NAD-dependent deacetylases (Grozinger et al. 2001), mammalian K⁺ channels (Zaks-Makhina et al. 2004), and mammalian p38 α MAP kinase (Friedmann et al. 2006).

Studies using *S. cerevisiae* first demonstrated the feasibility of cloning and studying mammalian PDE genes in a yeast system. *S. cerevisiae* expresses two PDE proteins, the Pde1 low-affinity enzyme, and the Pde2 high-affinity enzyme (Nikawa et al. 1987b; Sass et al. 1986). Cells lacking Pde1 and Pde2 have high cAMP levels that confer heat-shock sensitivity to stationary phase cells. Library screens for mammalian genes that restore heat-shock resistance led to the identification of clones of rat PDE4B and human PDE7A (Colicelli et al. 1989, 1991; Michaeli et al. 1993). In addition, yeast strains that express mammalian PDE4B display a heat-shock survival phenotype that is sensitive to treatment with the PDE4 specific inhibitor, rolipram (Engels et al. 1995; McHale et al. 1991; Pillai et al. 1993, 1994; Torphy et al. 1992). The ability to monitor PDE4 activity via heat shock resistance and the sensitivity of yeast-expressed rat PDE4B to rolipram allowed for the isolation of rolipram-resistant forms of PDE4B (Pillai et al. 1993). The amino acids altered in these proteins were later shown to be part of the nucleotide-binding site in the active site. While this system facilitated the cloning and study of PDEs, it is not amenable to high-throughput screening as abnormally high concentrations of rolipram are required for this assay. This may be due to the need to work with stationary phase cells that are relatively impenetrable to test compounds.

3 cAMP Signaling and *fbp1* Transcriptional Regulation in the Fission Yeast *S. pombe*

Both *S. pombe* and *S. cerevisiae* produce cAMP signals in response to glucose detection (D'Souza and Heitman 2001; Hoffman 2005a, b; Lengeler et al. 2000; Thevelein et al. 2000; Thevelein and de Winde 1999). In these yeasts, the increase in cAMP levels is due to adenylyl cyclase activation, while feedback regulation to

limit the cAMP signal is, in part, a function of PDE activity (Ma et al. 1999; Nikawa et al. 1987a; Wang et al. 2005).

Most of the components of the *S. pombe* cAMP pathway have been identified from mutant strains that are defective in glucose repression of transcription of the *fbp1* gene, which encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase (Hoffman and Winston 1990, 1991). Key to these genetic selections is a reporter construct in which the *fbp1* promoter drives expression of the *ura4* OMP decarboxylase gene. The *ura4* gene is a selectable marker as its expression is required for uracil biosynthesis, which is needed for growth in medium lacking uracil. It is also a counterselectable marker, as the pyrimidine analog 5-fluoroorotic acid (5FOA) is toxic to cells that express it. Normally, *S. pombe* represses *fbp1-ura4* transcription in glucose-rich medium, preventing growth in medium lacking uracil while allowing growth in 5FOA medium. Mutants defective in glucose/cAMP signaling, which is required to activate the cAMP-dependent protein kinase (PKA), constitutively transcribe the *fbp1-ura4* construct so as to confer growth on medium lacking uracil, as well as 5FOA-sensitivity (5FOA^S). These strains carry mutations that affect the Git3 G protein-coupled receptor (Welton and Hoffman 2000), any subunit of the heterotrimeric G protein composed of Gpa2 (Nocero et al. 1994), Git5 (Landry et al. 2000), and Git11 (Landry and Hoffman 2001), the Git2/Cyr1 adenylyl cyclase (Hoffman and Winston 1991), the Git1 adenylyl cyclase-binding protein (Kao et al. 2006), Hsp90 (Alaamery and Hoffman 2008), the Git7 Hsp90 co-chaperone (Schadick et al. 2002), or the Pka1 PKA catalytic subunit (Jin et al. 1995) (Fig. 1).

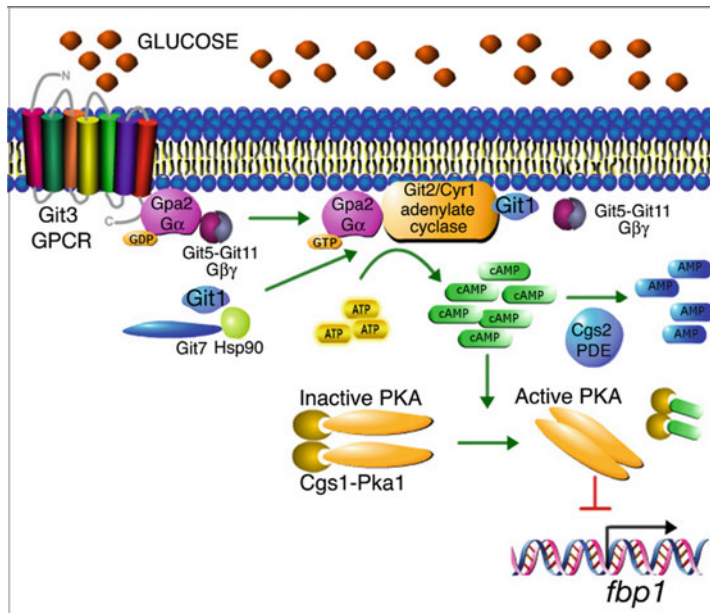


Fig. 1 The *S. pombe* glucose/cAMP signaling pathway presented using the ProteinLounge Pathway Builder Tool

Negative regulators of PKA were also identified using *fbp1-ura4* reporter-based screens. Mutations in the *cgs1* gene, which encodes the PKA regulatory subunit, were identified among suppressors of an adenylyl cyclase deletion (Stiefel et al. 2004), while mutations in the *cgs2* gene, which encodes the only *S. pombe* PDE, were identified among suppressors of an activation-defective form of adenylyl cyclase (Wang et al. 2005). In both selections, the *cgs1*⁻ and *cgs2*⁻ mutations restore 5FOA-resistant (5FOA^R) growth by repressing *fbp1-ura4* transcription. Thus, this system for studying *fbp1* transcriptional regulation is able to identify mutations that either reduce PKA activity to stimulate growth in medium lacking uracil or increase PKA activity to stimulate growth in 5FOA medium. The ability to use positive growth selections for mutations that either decrease or increase cAMP levels greatly enhances the robustness of these genetic selections. Although this system was initially used to identify mutations that alter PKA activity, we have recently deployed it as a HTS to detect small molecule inhibitors of heterologously expressed PDEs (Alaamery et al. 2010; Ivey et al. 2008). The screen capitalizes on the conversion of a PDE-expressing strain from 5FOA^S into 5FOA^R in the presence of a PDE inhibitor.

4 First Generation Screens

The cell growth-based screen to detect PDE inhibitors was developed by exploiting mutations that reduce glucose detection and cAMP synthesis. Glucose is sensed by the Git3 GPCR, which activates the Gpa2 G α subunit of the Gpa2-(G α) Git5-(G β) Git11-(G γ) heterotrimeric G protein. Gpa2-mediated activation of adenylyl cyclase produces a cAMP signal to activate PKA, which represses *fbp1* transcription by multiple mechanisms (Fig. 1). Deletion of any of the *git3*, *gpa2*, *git5*, or *git11* genes confers a 5FOA^S phenotype due to increased expression of the *fbp1-ura4* reporter (Landry and Hoffman 2001; Landry et al. 2000; Nocero et al. 1994; Welton and Hoffman 2000). Assays of *fbp1-lacZ* expression in these strains reveal that the relative importance of these proteins in glucose repression is Gpa2, Git3, Git5, and Git11. Loss of Cgs2 PDE activity restores 5FOA^R growth and repression of *fbp1-lacZ* expression in all of these mutants, providing the conceptual basis for PDE inhibitor screens.

The key to developing a strain suitable for a PDE inhibitor HTS lies in the interaction between the heterologous PDE and the endogenous cAMP signaling pathway. The PDE must lower the intracellular cAMP level sufficiently to create a 5FOA^S growth phenotype that would be converted to 5FOA^R upon inhibition of the PDE. For highly active PDEs, little or no additional engineering is necessary to attain 5FOA sensitivity; for example, strains expressing either of two isoforms of the highly active human PDE4D enzyme (Conti and Beavo 2007; Houslay et al. 2007) display a leaky 5FOA^S in cells with an intact cAMP signaling pathway and require the loss of only the Git11 G γ to produce a tight 5FOA^S phenotype. Strains expressing PDE4A or PDE4B enzymes encoded by two other genes of the PDE4 family, or PDE7A or PDE7B high-affinity cAMP-specific enzymes (Bender and

Beavo 2006; Conti and Beavo 2007) require more severe disabling of cAMP signaling conferred by the loss of the Gpa2 G α to produce a tight 5FOA^S phenotype. Loss of Gpa2 is not sufficient to confer 5FOA^S growth in strains expressing the cAMP-specific IBMX-insensitive PDE8A enzyme (Bender and Beavo 2006; Conti and Beavo 2007), suggesting very low activity of this enzyme when expressed in *S. pombe* (in these strains, the full-length mammalian genes are expressed from the yeast PDE gene promoter at its genomic locus). The 5FOA^S strains are suitable for HTSs for PDE inhibitors that elevate cAMP levels and thus repress *fbp1-ura4* transcription to allow 5FOA^R growth (Fig. 2). However, this approach is not amenable for screening strains expressing PDEs such as PDE8A whose activity is insufficient to confer 5FOA^S growth in these mutant backgrounds.

HTSs were optimized using a 384 well format. Assay development involves optimizing growth conditions prior to screening to prevent *ura4* reporter expression (thus PDE inhibitors maintain, rather than establish, repression of the reporter) and initial cell density. A 48-h incubation period is required for actively growing strains to reach saturation, allowing the greatest contrast in optical density between vehicle-treated cultures (OD₆₀₀ = 0.05–0.2) and PDE inhibitor-treated or cAMP-treated cultures that reach a saturated cell density (OD₆₀₀ = 1.2–1.3). Optimized assays generally produce Z-factors of 0.7–0.9, indicative of highly robust screens.

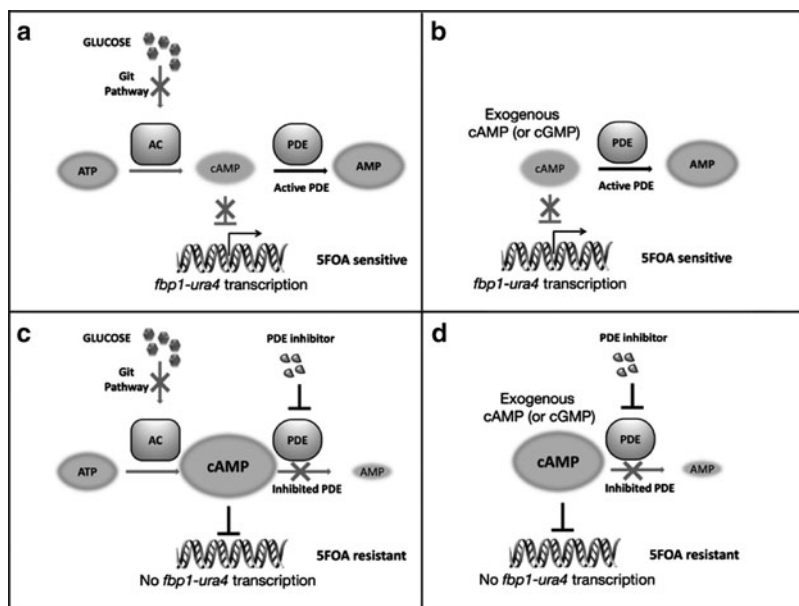
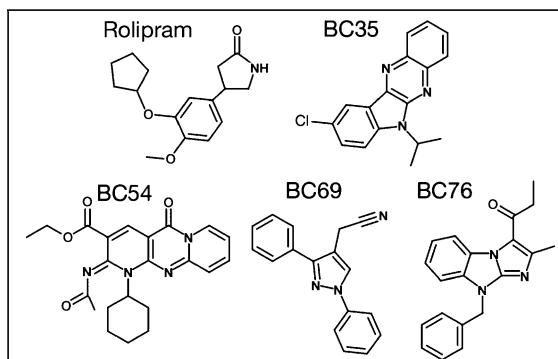


Fig. 2 First, second and third generation 5FOA-growth screens. (a) 5FOA^S growth in a first generation screening strain is due to a defective glucose-sensing Git pathway. (b) 5FOA^S growth in a second or third generation screening strain is due to PDE degradation of exogenously added cAMP or cGMP. (c, d) PDE inhibition elevates intracellular cAMP or cGMP levels to repress *fbp1-ura4* transcription and confer 5FOA^R growth

Fig. 3 Structures of rolipram and four PDE inhibitors identified in HTSs using strains expressing PDE4 or PDE7 enzymes



HTSs using strains that express mouse PDE4A1 (Cherry et al. 2001), rat PDE4A5 (McPhee et al. 1995), mouse PDE4B3 (Cherry et al. 2001), human PDE7A1 (Michaeli et al. 1993), and *S. pombe* Cgs2 (DeVoti et al. 1991) were carried out at the Broad Institute's screening facility (Alaamery et al. 2010; Ivey et al. 2008). Of ~74,000 compounds screened at ~20 μ M in duplicate using a strain expressing PDE4A1, the PDE4 selective inhibitor rolipram (Wachtel 1982) ranked 34th and the PDE3/4 inhibitor zardaverine (Schudt et al. 1991) ranked 27th by Composite Z score. Ninety-one compounds produced average OD₆₀₀ values of >1.2, which represent cultures grown to near saturation. The ability of these compounds to stimulate this level of growth suggests that they are permeable to the yeast cells, not toxic to yeast, and remain active for most or all of the 48-h incubation period. Low toxicity serves as a proxy for selectivity, since compounds that bind to many proteins would likely inhibit some essential yeast proteins and prevent cell growth. The relative potencies of the compounds that confer saturated growth cannot be determined from these data alone.

To examine potency as judged by this assay, dose–response curves were carried out to measure the ability of compounds, such as those shown in Fig. 3, to stimulate cell growth at <20 μ M (the concentration used in the initial screens). As seen in Fig. 4, rolipram increases cell growth of a strain expressing human PDE4B2 (Bolger et al. 1993) to OD₆₀₀ = 0.6 (half that of a saturated culture) when present at <2 μ M, while some structurally diverse compounds identified in these screens, such as BC35 (identified in the PDE4 screens) and BC54 (identified in PDE4 and PDE7 screens), are effective at significantly lower concentrations. Therefore, this assay is very sensitive with regard to inhibitor detection. In vitro enzyme assays confirm the relative potencies of these PDE inhibitors.

5 Second Generation Screens

Second generation screening strains were developed to allow screening for inhibitors of enzymes such as PDE8A (Fisher et al. 1998a) for which the activity is insufficient to confer 5FOA^S growth even in strains lacking the Gpa2 G α . Unlike

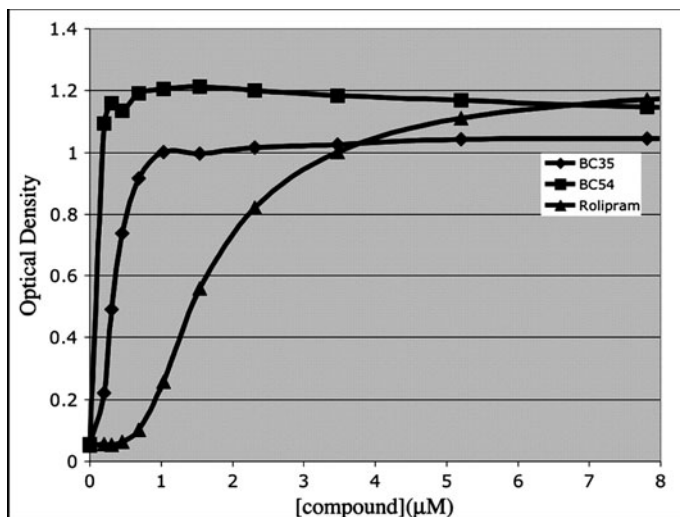


Fig. 4 Effect of rolipram, BC35, and BC54 (structures shown in Fig. 3) on 5FOA^R growth of a PDE4B2 expressing strain. OD₆₀₀ values were measured in the presence of various concentrations of compounds in 384 well microtiter dishes after 48-h growth

the budding yeast *S. cerevisiae*, *S. pombe* strains that lack either adenylyl cyclase or PKA are viable. Deletion of the *git2* adenylyl cyclase gene abolishes cAMP synthesis, so that PKA can be regulated by addition of cAMP to the growth medium. The expressed PDE activity reduces cell growth in response to exogenous cAMP (Fig. 2), allowing one to determine the optimal concentration of cAMP to add to the medium for inhibitor detection. For PDE8A-expressing cells, 40 μM cAMP is optimal for achieving differential growth in 5FOA medium, as this level of cAMP stimulates growth to OD₆₀₀ >1.0 of a strain lacking PDE activity, while having little effect on a PDE8A-expressing strain (Fig. 5). These conditions were used to measure PDE8A inhibition by 56 PDE4 or PDE7 inhibitors identified from the first generation screens and revealed that the PDE4 inhibitor BC69 (Fig. 3) is an effective inhibitor of PDE8A (Fig. 5). Further work with BC69 allowed the optimization of a PDE8A inhibitor HTS.

6 Third Generation Screens

The screens described above exploit the fact that the expressed PDE can hydrolyze endogenously produced or exogenously added cAMP to reduce PKA activity. As such, these screens detect inhibitors of cAMP hydrolysis. We have found that *S. pombe* PKA can also be activated by exogenously-added

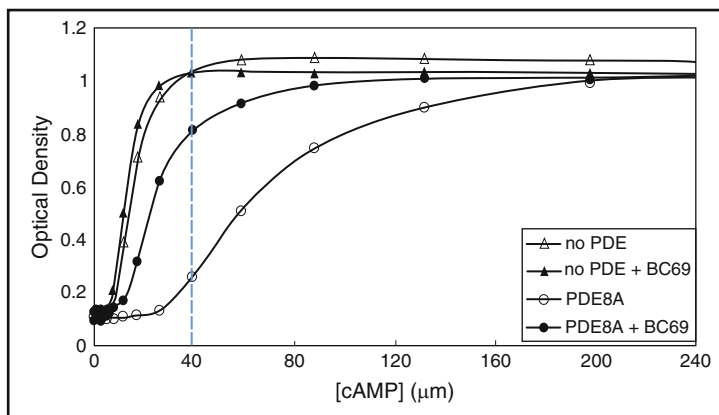


Fig. 5 5FOA^R growth conferred by exogenous cAMP on strains lacking adenylyl cyclase. OD₆₀₀ values were determined for strains lacking adenylyl cyclase and either lacking PDE activity or expressing PDE8A. The X-axis indicates the concentration of cAMP (μM) added to the 5FOA medium. At 40 μM cAMP (vertical dashed line), the OD₆₀₀ for the strain lacking PDE activity is fivefold higher than for the strain expressing PDE8A. Addition of 20 μM BC69 (Fig. 3; identified in a PDE4 inhibitor screen) shifts the cAMP response curve of the PDE8A-expressing strain toward that of the strain lacking PDE activity

cGMP, promoting 5FOA^R growth of a strain that lacks both adenylyl cyclase and PDE activity (Fig. 6). Similar to the second-generation screens, cGMP stimulated growth is reduced upon expression of a PDE that hydrolyzes cGMP. This allows for screens to detect inhibitors of cGMP-specific PDEs, while inhibitors of dual specificity PDEs can be screened for using exogenous cAMP or cGMP (Fig. 2).

The ability to use exogenous cGMP for screening was validated using strains expressing cGMP-specific PDE5A (McAllister-Lucas et al. 1993) and PDE9A (Fisher et al. 1998b). As expected for a strain lacking both adenylyl cyclase and PDE activity, a higher concentration of cGMP is required to stimulate growth relative to cAMP. 40 μM cGMP produces an OD₆₀₀ = 0.6 as compared to 12.5 μM cAMP, while 100 μM cGMP results in saturated growth as compared to 25 μM cAMP. Expression of either PDE5A or PDE9A completely eliminates the response to cGMP at these, as well as significantly higher, concentrations. As described above for PDE8A, a screen of inhibitors obtained in either PDE4 or PDE7 HTSs led to the identification of compound BC76 (Fig. 3) as an effective PDE5A inhibitor. In vitro enzyme assays confirm that BC76 is a PDE5A inhibitor with an IC₅₀ of ~250 nM (Sharron Francis, personal communication).

These three iterations of screening strains and methods have allowed us to construct strains that express mammalian PDEs from 10 of the 11 PDE families (with the exception of the PDE6 family of the visual system), all of which display sufficient activity to permit inhibitor screens based on 5FOA^R growth.

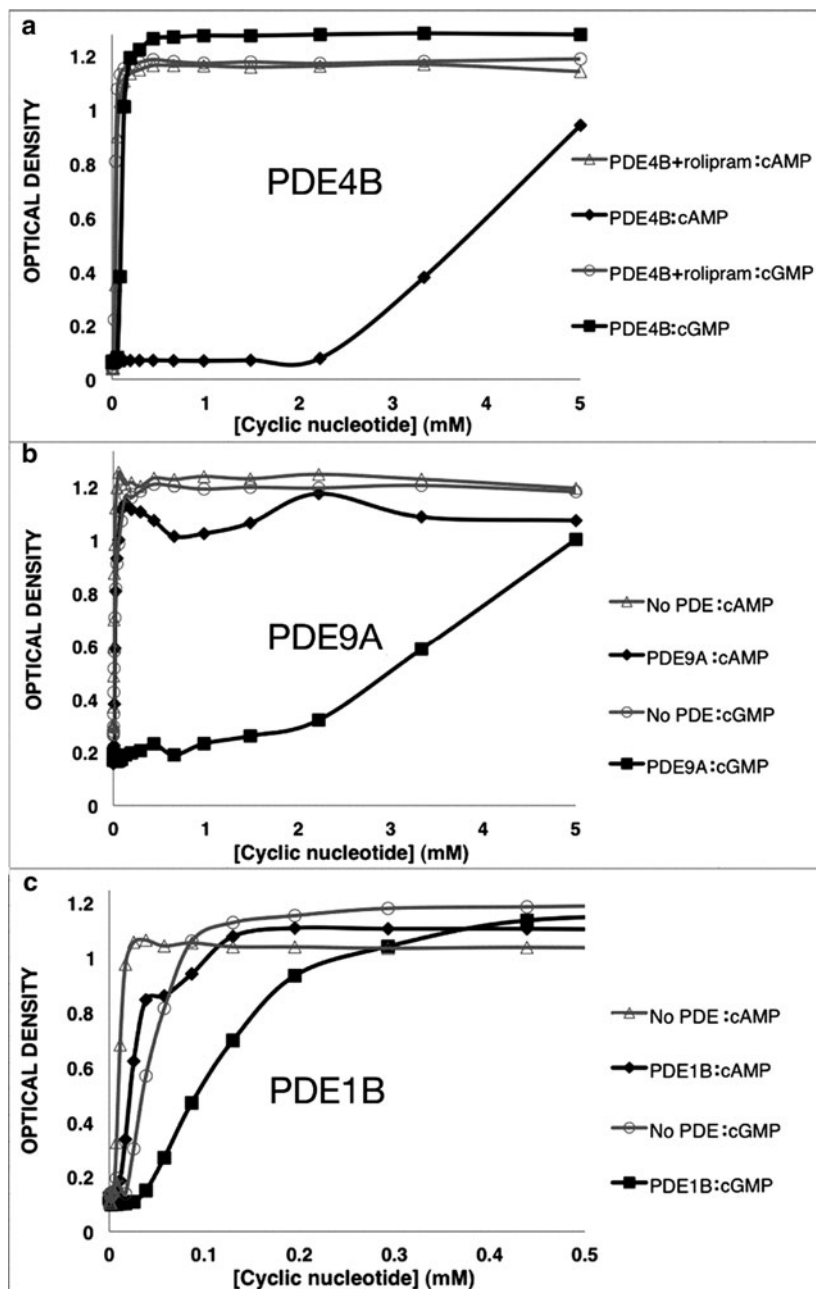


Fig. 6 cAMP/cGMP profiles for PDE4B, PDE9A, and PDE1B. See text for details

7 Profiling PDE Activity in Strains Lacking Adenylyl Cyclase

The expression of PDEs in strains lacking adenylyl cyclase provides a favorable context for observing the properties of PDEs in live cells (although to be sure, *S. pombe* lacks many of the PDE-interacting proteins found in mammalian cells). By comparing the cyclic nucleotide-mediated growth response of a strain that lacks both adenylyl cyclase and PDE activity to that of a PDE-expressing strain, one can assess the relative activity of the PDE against cAMP and cGMP (Fig. 6). We see, for example, that a strain expressing PDE4B requires a final concentration of ~4 mM cAMP in the medium to stimulate cell growth to $OD_{600} = 0.6$, while the same strain grown in the presence of 40 μ M rolipram (Fig. 6a) or a strain lacking PDE activity (Fig. 6b, c) requires <50 μ M cAMP in the medium to reach this OD_{600} . In contrast, expression of PDE4B shifts the concentration of cGMP needed to stimulate growth by only ~60 μ M (Fig. 6a), although this may represent enough cGMP hydrolyzing activity by PDE4B to play a role in controlling cGMP levels in certain mammalian cells. A strain expressing the cGMP-specific PDE9A requires >3 mM more cGMP to stimulate growth to $OD_{600} = 0.6$ than does a strain lacking PDE activity, while these strains show identical responses to cAMP (Fig. 6b). A strain expressing the dual cAMP/cGMP hydrolyzing PDE1B enzyme (Repaske et al. 1992) requires 10 μ M more cAMP or 70 μ M more cGMP in the medium to stimulate growth to $OD_{600} = 0.6$ than does a strain lacking PDE activity (Fig. 6c). Low activity of mammalian calcium/calmodulin-activated PDE1B expressed in *S. pombe* could be because *S. pombe* calmodulin is only 74% identical to mammalian calmodulin and may be unable to activate PDE1B.

8 Additional Uses of this System

The reciprocal growth behavior of strains expressing the *fbp1-ura4* reporter in medium containing 5FOA (in which high cAMP levels are required for growth) versus medium lacking uracil (in which low cAMP levels are required for growth), allows for screens of PDE activators as well as PDE inhibitors. While such compounds should be detectable by their ability to promote growth of strains with high cAMP levels in medium lacking uracil, two pilot screens failed to identify compounds that activated either PDE7A or PDE8A. This may be due to the fact that PDE activators will be much less common than PDE inhibitors, just as dominant gain-of-function mutations are less common than recessive loss-of-function mutations. The reciprocal growth behavior in medium lacking uracil versus 5FOA medium conferred by the *fbp1-ura4* reporter could also be used to facilitate growth enrichment and genetic screens for strains expressing mutant alleles of PDE genes that encode inhibitor-resistant enzymes. The ability of *S. pombe* to maintain autonomous plasmids allows for the screening of cDNA libraries, cloned in

S. pombe expression vectors, for either PDE-activating or inhibiting proteins. Stably incorporating genes expressing such proteins into PDE-expressing strains would facilitate genetic studies of PDE regulator activity or small molecule screens similar to those described above. In summary, the introduction of PDEs (which could include those from nonmammalian sources) into *S. pombe* strains allows for a wide variety of classical and chemical genetic screens to enhance our understanding of these enzymes, and to advance the discovery of chemical tools and the development of therapeutic compounds.

9 Conclusions

Three generations of strain development has produced a *S. pombe*-based screening platform that can be used to detect inhibitors of a wide range of full-length PDEs expressed in a eukaryotic cell. The compounds detected in this manner are stable, cell permeable, and of low toxicity to *S. pombe*, characteristics that may make them attractive candidates for drug development. In addition to facilitating new inhibitor discovery and drug development, this system can be used to characterize PDEs and to study PDEs together with biological regulators in a heterologous system that is amenable to detecting subtle changes in PDE activity.

References

- Alaamery MA, Hoffman CS (2008) *Schizosaccharomyces pombe* Hsp90/Git10 is required for glucose/cAMP signaling. *Genetics* 178:1927–1936
- Alaamery MA, Wyman AR, Ivey FD, Allain C, Demirbas D, Wang L, Ceyhan O, Hoffman CS (2010) New classes of PDE7 inhibitors identified by a fission yeast-based HTS. *J Biomol Screen* 15(4):359–367
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Bischoff JR, Casso D, Beach D (1992) Human p53 inhibits growth in *Schizosaccharomyces pombe*. *Mol Cell Biol* 12:1405–1411
- Bolger G, Michaeli T, Martins T, St John T, Steiner B, Rodgers L, Riggs M, Wigler M, Ferguson K (1993) A family of human phosphodiesterases homologous to the dunce learning and memory gene product of *Drosophila melanogaster* are potential targets for antidepressant drugs. *Mol Cell Biol* 13:6558–6571
- Card GL, England BP, Suzuki Y, Fong D, Powell B, Lee B, Luu C, Tabrizid M, Gillette S, Ibrahim PN, Artis DR, Bollag G, Milburn MV, Kim SH, Schlessinger J, Zhang KY (2004) Structural basis for the activity of drugs that inhibit phosphodiesterases. *Structure* 12:2233–2247
- Card GL, Blasdel L, England BP, Zhang C, Suzuki Y, Gillette S, Fong D, Ibrahim PN, Artis DR, Bollag G, Milburn MV, Kim SH, Schlessinger J, Zhang KY (2005) A family of phosphodiesterase inhibitors discovered by cocrystallography and scaffold-based drug design. *Nat Biotechnol* 23:201–207

- Cherry JA, Thompson BE, Pho V (2001) Diazepam and rolipram differentially inhibit cyclic AMP-specific phosphodiesterases PDE4A1 and PDE4B3 in the mouse. *Biochim Biophys Acta* 1518:27–35
- Colicelli J, Birchmeier C, Michaeli T, O'Neill K, Riggs M, Wigler M (1989) Isolation and characterization of a mammalian gene encoding a high-affinity cAMP phosphodiesterase. *Proc Natl Acad Sci USA* 86:3599–3603
- Colicelli J, Nicolette C, Birchmeier C, Rodgers L, Riggs M, Wigler M (1991) Expression of three mammalian cDNAs that interfere with RAS function in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 88:2913–2917
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- D'Souza CA, Heitman J (2001) Conserved cAMP signaling cascades regulate fungal development and virulence. *FEMS Microbiol Rev* 25:349–364
- DeVoti J, Seydoux G, Beach D, McLeod M (1991) Interaction between ran1⁺ protein kinase and cAMP dependent protein kinase as negative regulators of fission yeast meiosis. *EMBO J* 10:3759–3768
- Engels P, Sullivan M, Muller T, Lubbert H (1995) Molecular cloning and functional expression in yeast of a human cAMP-specific phosphodiesterase subtype (PDE IV-C). *FEBS Lett* 358:305–310
- Fisher DA, Smith JF, Pillar JS, St Denis SH, Cheng JB (1998a) Isolation and characterization of PDE8A, a novel human cAMP-specific phosphodiesterase. *Biochem Biophys Res Commun* 246:570–577
- Fisher DA, Smith JF, Pillar JS, St Denis SH, Cheng JB (1998b) Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. *J Biol Chem* 273:15559–15564
- Friedmann Y, Shriki A, Bennett ER, Golos S, Diskin R, Marbach I, Bengal E, Engelberg D (2006) JX401, A p38alpha inhibitor containing a 4-benzylpiperidine motif, identified via a novel screening system in yeast. *Mol Pharmacol* 70:1395–1405
- Grozinger CM, Chao ED, Blackwell HE, Moazed D, Schreiber SL (2001) Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. *J Biol Chem* 276:38837–38843
- Hoffman CS (2005a) Except in every detail: comparing and contrasting G protein signaling in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Eukaryot Cell* 4:495–503
- Hoffman CS (2005b) Glucose sensing via the protein kinase A pathway in *Schizosaccharomyces pombe*. *Biochem Soc Trans* 33:257–260
- Hoffman CS, Winston F (1990) Isolation and characterization of mutants constitutive for expression of the *fbp1* gene of *Schizosaccharomyces pombe*. *Genetics* 124:807–816
- Hoffman CS, Winston F (1991) Glucose repression of transcription of the *Schizosaccharomyces pombe fbp1* gene occurs by a cAMP signaling pathway. *Genes Dev* 5:561–571
- Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 35:91–100
- Houslay MD, Baillie GS, Maurice DH (2007) cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. *Circ Res* 100:950–966
- Ivey FD, Wang L, Demirbas D, Allain C, Hoffman CS (2008) Development of a fission yeast-based high-throughput screen to identify chemical regulators of cAMP phosphodiesterases. *J Biomol Screen* 13:62–71
- Jin M, Fujita M, Culley BM, Apolinario E, Yamamoto M, Maundrell K, Hoffman CS (1995) *sck1*, a high copy number suppressor of defects in the cAMP-dependent protein kinase pathway in fission yeast, encodes a protein homologous to the *Saccharomyces cerevisiae* SCH9 kinase. *Genetics* 140:457–467
- Kao RS, Morreale E, Wang L, Ivey FD, Hoffman CS (2006) *Schizosaccharomyces pombe* Git1 is a C2-domain protein required for glucose activation of adenylate cyclase. *Genetics* 173:49–61
- Landry S, Hoffman CS (2001) The git5 G β and git11 G γ form an atypical G $\beta\gamma$ dimer acting in the fission yeast glucose/cAMP pathway. *Genetics* 157:1159–1168

- Landry S, Pettit MT, Apolinario E, Hoffman CS (2000) The fission yeast *git5* gene encodes a G β subunit required for glucose-triggered adenylate cyclase activation. *Genetics* 154:1463–1471
- Lee MG, Nurse P (1987) Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature* 327:31–35
- Lengeler KB, Davidson RC, D'Souza C, Harashima T, Shen WC, Wang P, Pan X, Waugh M, Heitman J (2000) Signal transduction cascades regulating fungal development and virulence. *Microbiol Mol Biol Rev* 64:746–785
- Lerner A, Epstein PM (2006) Cyclic nucleotide phosphodiesterases as targets for treatment of haematological malignancies. *Biochem J* 393:21–41
- Ma P, Wera S, Van Dijck P, Thevelein JM (1999) The *PDE1*-encoded low-affinity phosphodiesterase in the yeast *Saccharomyces cerevisiae* has a specific function in controlling agonist-induced cAMP signaling. *Mol Biol Cell* 10:91–104
- McAllister-Lucas LM, Sonnenburg WK, Kadlecak A, Seger D, Trong HL, Colbran JL, Thomas MK, Walsh KA, Francis SH, Corbin JD et al (1993) The structure of a bovine lung cGMP-binding, cGMP-specific phosphodiesterase deduced from a cDNA clone. *J Biol Chem* 268:22863–22873
- McHale MM, Cieslinski LB, Eng WK, Johnson RK, Torphy TJ, Livi GP (1991) Expression of human recombinant cAMP phosphodiesterase isozyme IV reverses growth arrest phenotypes in phosphodiesterase-deficient yeast. *Mol Pharmacol* 39:109–113
- McPhee I, Pooley L, Lobban M, Bolger G, Houslay MD (1995) Identification, characterization and regional distribution in brain of RPDE-6 (RNPDE4A5), a novel splice variant of the PDE4A cyclic AMP phosphodiesterase family. *Biochem J* 310(Pt 3):965–974
- Michaeli T, Bloom TJ, Martins T, Loughney K, Ferguson K, Riggs M, Rodgers L, Beavo JA, Wigler M (1993) Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase-deficient *Saccharomyces cerevisiae*. *J Biol Chem* 268:12925–12932
- Nikawa J, Cameron S, Toda T, Ferguson KM, Wigler M (1987a) Rigorous feedback control of cAMP levels in *Saccharomyces cerevisiae*. *Genes Dev* 1:931–937
- Nikawa J, Sass P, Wigler M (1987b) Cloning and characterization of the low-affinity cyclic AMP phosphodiesterase gene of *Saccharomyces cerevisiae*. *Mol Cell Biol* 7:3629–3636
- Nocero M, Isshiki T, Yamamoto M, Hoffman CS (1994) Glucose repression of *fbp1* transcription of *Schizosaccharomyces pombe* is partially regulated by adenylate cyclase activation by a G protein α subunit encoded by *gpa2* (*git8*). *Genetics* 138:39–45
- Pillai R, Kytel K, Reyes A, Colicelli J (1993) Use of a yeast expression system for the isolation and analysis of drug-resistant mutants of a mammalian phosphodiesterase. *Proc Natl Acad Sci USA* 90:11970–11974
- Pillai R, Staub SF, Colicelli J (1994) Mutational mapping of kinetic and pharmacological properties of a human cardiac cAMP phosphodiesterase. *J Biol Chem* 269:30676–30681
- Repaske DR, Swinnen JV, Jin SL, Van Wyk JJ, Conti M (1992) A polymerase chain reaction strategy to identify and clone cyclic nucleotide phosphodiesterase cDNAs. Molecular cloning of the cDNA encoding the 63-kDa calmodulin-dependent phosphodiesterase. *J Biol Chem* 267:18683–18688
- Sass P, Field J, Nikawa J, Toda T, Wigler M (1986) Cloning and characterization of the high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 83:9303–9307
- Schadick K, Fourcade HM, Boumenot P, Seitz JJ, Morrell JL, Chang L, Gould KL, Partridge JF, Allshire RC, Kitagawa K, Hieter P, Hoffman CS (2002) *Schizosaccharomyces pombe* *Git7p*, a member of the *Saccharomyces cerevisiae* *Sgt1p* family, is required for glucose and cyclic AMP signaling, cell wall integrity, and septation. *Eukaryot Cell* 1:558–567
- Schudt C, Winder S, Eltze M, Kilian U, Beume R (1991) Zardaverine: a cyclic AMP specific PDE III/IV inhibitor. *Agents Actions Suppl* 34:379–402
- Soderling SH, Beavo JA (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Curr Opin Cell Biol* 12:174–179

- Stiefel J, Wang L, Kelly DA, Janoo RTK, Seitz J, Whitehall SK, Hoffman CS (2004) Suppressors of an adenylate cyclase deletion in the fission yeast *Schizosaccharomyces pombe*. *Eukaryot Cell* 3:610–619
- Thevelein JM, de Winde JH (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* 33:904–918
- Thevelein JM, Cauwenberg L, Colombo S, De Winde JH, Donation M, Dumortier F, Kraakman L, Lemaire K, Ma P, Nauwelaers D, Rolland F, Teunissen A, Van Dijck P, Versele M, Wera S, Winderickx J (2000) Nutrient-induced signal transduction through the protein kinase A pathway and its role in the control of metabolism, stress resistance, and growth in yeast. *Enzyme Microb Technol* 26:819–825
- Torphy TJ, Stadel JM, Burman M, Cieslinski LB, McLaughlin MM, White JR, Livi GP (1992) Coexpression of human cAMP-specific phosphodiesterase activity and high affinity rolipram binding in yeast. *J Biol Chem* 267:1798–1804
- Wachtel H (1982) Characteristic behavioural alterations in rats induced by rolipram and other selective adenosine cyclic 3', 5'-monophosphate phosphodiesterase inhibitors. *Psychopharmacol (Berl)* 77:309–316
- Wang L, Griffiths K, Zhang YH, Ivey FD, Hoffman CS (2005) *Schizosaccharomyces pombe* adenylate cyclase suppressor mutations suggest a role for cAMP phosphodiesterase regulation in feedback control of glucose/cAMP signaling. *Genetics* 171:1523–1533
- Welton RM, Hoffman CS (2000) Glucose monitoring in fission yeast via the *gpa2* G α , the *git5* G β , and the *git3* putative glucose receptor. *Genetics* 156:513–521
- Zaks-Makhina E, Kim Y, Aizenman E, Levitan ES (2004) Novel neuroprotective K⁺ channel inhibitor identified by high-throughput screening in yeast. *Mol Pharmacol* 65:214–219

The GAF-Tandem Domain of Phosphodiesterase 5 as a Potential Drug Target

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and Gabriele Sorg

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Abstract Classic PDE5 inhibitors interact with and block the catalytic site of PDE5. They have been clinically validated for treatment of erectile dysfunction as well as reduction of pulmonary arterial pressure, improvement of exercise capacity, quality of life, and arterial oxygenation in patients with secondary pulmonary hypertension. Minor side effects are visual disturbances, headache, migraine, back pain, and interaction with nitrates (hypotension). Some of those side effects presumably can be ameliorated by improving selectivity and pharmacokinetics; other side effects probably are target related due to inhibition of basic physiological processes. Target related side effects may be bypassed by using PDE5 inhibitors with a different mode of action: PDE5, like PDE2, PDE6, PDE10, and PDE11, is a multidomain protein with an N-terminal tandem GAF domain, which in case of PDE5, is allosterically activated by cGMP. Potential inhibitors acting at the PDE5 GAF domain would be expected to inhibit only pathophysiologically upregulated PDE5 activity, whereas basal activity of PDE5 would remain unaffected.

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Here, we summarize a high-throughput screening campaign to identify inhibitors of the regulatory GAF domain of human PDE5. To target the regulatory domain independently from the catalytic site, we used a chimeric reporter enzyme: The hPDE5 GAF-tandem domain functionally replaced the GAF domain in the cyanobacterial adenylyl cyclase CyaB1. We identified inhibitors that target the GAF domain and also inhibitors that target the bacterial cyclase.

Compounds binding to the PDE5 GAF domain were reanalysed with native human PDE5 to demonstrate inhibition using capillary electrophoresis. This identified 16 compounds that act on the GAF domain of PDE5. Two compounds fulfilled the initial requirement to inhibit, exclusively, activated PDE5, but not basal PDE5 activity.

Keywords Adenylyl cyclase · Drug target · GAF domain · High-throughput screen · Phosphodiesterase 5 inhibitor

Abbreviations

cAMP	3',5'-cyclic adenosine monophosphate
cGMP	3',5'-cyclic guanosine monophosphate
hPDE	Human phosphodiesterase
PDE	Phosphodiesterase
SD	Standard deviation

1 Introduction and Scope

For decades, 3',5' cyclic nucleotide monophosphate phosphodiesterases (PDEs) have been valued as potential drug targets (Lugnier 2006; Manallack et al. 2005; Menniti et al. 2006; Omori and Kotera 2007; Rose et al. 2005; Rotella 2002; Vasta and Beavo 2004), with, at the beginning of the 1970s in the last century, several drug companies initiating programs in this field (Bergstrand et al. 1977; Chasin and Harris 1976; Francis et al. 2001). At that time, evidence had accumulated that several PDE forms exist; yet their molecular relationship and complexity was unknown. With the advent of molecular biology, we now know that 11 mammalian PDE families exist that are encoded by 21 genes (Bender and Beavo 2006; Francis et al. 2001). Multiple alternative splicing of these genes generates additional PDE isoforms (Zoraghi et al. 2004). Currently, the number of identified PDE splice variants exceeds 100 (Conti and Beavo 2007; Omori and Kotera 2007). It is tempting to ask whether each of these “in vitro” identified gene products will serve a specific physiological role in the regulation of cellular signaling processes.

So far, with the potential exception of the photoreceptor PDE6, there is evidence indicating that all mammalian PDEs can form homodimers, albeit the recombinant catalytic domains are active as monomers (Stroop and Beavo 1991). Insight into

this discrepancy was recently provided with the publication of the structure of the human PDE2A holoenzyme, which is a dimer (Pandit et al. 2009). This structure has provided novel and exciting insights into our understanding of the regulation of PDE2 activity. It demonstrated that, in the homodimer, the catalytic domains of PDE2 occlude each other, thus impeding access of substrate to the catalytic site. PDE2 has an N-terminal tandem GAF domain, GAF-A and GAF-B. Binding of cGMP to GAF-B in PDE2 is proposed to trigger rotation of the monomers against each other and, thereby, open the catalytic sites for substrate binding, i.e., the catalytic center is active as a monomer and dimerization is an instrument for regulation by controlling the position of the catalytic domains and solvent access of the catalytic sites (Pandit et al. 2009). Albeit it is debatable whether the insights with human PDE2 can be extended across different PDE families, this certainly provides a very attractive hypothesis considering the similarity of several architectural features in mammalian PDEs (Beavo and Brunton 2002; Conti and Beavo 2007; Francis et al. 2001).

The variety of different PDEs recognized during the last two decades has resulted in a renaissance for PDEs as drug targets. Probably, we are only at the beginning of exploiting this potential as just a few family-specific PDE inhibitors have reached the market and a few others are in clinical development (Aversa et al. 2006; Croom and Curran 2008; Houslay et al. 2005; Wang et al. 2007). The suitability of PDEs as drug targets appears to be due to several prominent features. (1) Twenty-one distinct gene families exist, which share certain structural similarities – regulatory domains generally are located toward the N-terminus and the catalytic domains are located toward the C-terminus (Conti and Beavo 2007; Handa et al. 2008); regulatory and catalytic domains of different PDE families have distinct molecular properties, and hence, may be amenable to development of specific drugs; (2) within individual PDE families several distinctive isoforms exist, which appear either differentially regulated or localized to peculiar subcellular compartments or multiprotein complexes (Houslay 2010); (3) the X-ray structures of most catalytic PDE domains (exceptions are PDE6 and 11) show defined structural variations and the development of rather specific inhibitors of individual catalytic domains appears possible (Conti and Beavo 2007; Pandit et al. 2009; Zhang et al. 2005). In fact, a number of compounds have been designed and developed, which show a very high degree of PDE subfamily specificity (Lugnier 2006). Enzymatic assays using recombinant catalytic domains that are very active as monomers have been adapted to an industrial high-throughput format. Complications that may arise with the multi-domain PDE holoenzymes are thereby effectively circumnavigated.

So far, the regulatory domains of PDEs have not been described as drug targets, although, in principle, they should be quite attractive. PDE2, PDE5, PDE6, PDE10, and PDE11 contain an N-terminal tandem GAF domain that, upon binding of cGMP (cAMP for PDE10), enhances catalytic activity (see above). The acronym GAF is derived from the first identified GAF proteins, namely mammalian cGMP-regulated PDEs, *Anabaena* adenylyl cyclase, and the *E. coli* transcription factor Fhl A (Aravind and Ponting 1997). On the other hand, PDE1 isoforms that are encoded by three separate genes possess an N-terminal tandem of two calmodulin-binding

domains, and long isoforms in the PDE4 family, encoded by four separate genes, have a tandem of two UCR domains N-terminal to the catalytic domain (the acronym UCR stands for upstream-conserved region), and phosphorylation in this region augments PDE4 activity (Houslay and Adams 2003; Sette and Conti 1996). However, the biochemical characterization of the relationship between regulatory and catalytic domains remains cumbersome. For PDEs 2, 5, 6, 10, and 11, the allosteric activator cGMP (cAMP for PDE10) concomitantly serves as a substrate. This seriously limits the kinetic characterization and renders the design of high-throughput assays for modulators of these allosteric sites difficult. In 2002, we demonstrated that the GAF-tandem domain of PDE2 is functionally interchangeable with the GAF-tandem domain from the cyanobacterial adenylyl cyclase CyaB1, i.e., the adenylyl cyclase, which intrinsically is regulated by its enzymatic product cAMP, is turned into a cGMP-regulated adenylyl cyclase by appending the region of PDE2 that contains the tandem GAF domains (Kanacher et al. 2002). Activation is robust and the biochemical properties of the tandem GAF domain in the chimera reflect those reported in studies of recombinant isolated GAF domains or in conjunction with its catalytic PDE domain (Corbin et al. 2000, 2003). Several subsequent studies involving the tandem GAF domains from PDE5, PDE10, and PDE11 in chimeras with the cyanobacterial cyclase have generally confirmed that the cyclase can be used as an excellent reporter enzyme to characterize biochemical properties of mammalian PDE GAF-tandem domains (Bruder et al. 2006; Gross-Langenhoff et al. 2006; Hofbauer et al. 2008). In fact, this has been extended to the tandem calmodulin-binding domains from PDE1 and the tandem UCR domains from PDE4 isoforms (Banjac and Schultz, unpublished). The constructs with the tandem GAF domains offer the advantage that the allosteric activator/regulator and enzyme substrate are separate, noninterfering chemicals.

Here, we describe the use of a chimeric protein comprising the tandem GAF domain of human PDE5 (hPDE5) fused to the adenylyl cyclase CyaB1. We have exploited this novel construct for use in a high-throughput screen of a diverse chemical library with several 100,000 compounds to identify substances that might inhibit adenylyl cyclase activation via interaction with the regulatory tandem GAF domains from hPDE5. Here, we critically review the assay parameters and the feasibility of such a large-scale assay.

2 Requirements to Run a High-Throughput Screen

The PDE5 tandem GAF–CyaB1 adenylyl cyclase chimera (Fig. 1; PDE5-GAF–CyaB1) converts ATP to equimolar amounts of cAMP and pyrophosphate. A key question in developing a high-throughput screen involves determining which product of the enzymatic reaction can be measured in a simple, reliable, quick, and cost-effective way cAMP determination via ELISA, although well established, is costly and due to several washing steps unsuitable for automation. Sensitive colorimetric methods to determine inorganic phosphate using ammonium molybdate and

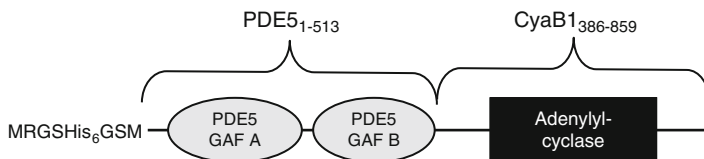


Fig. 1 Construct design of chimeric protein PDE5-GAF-CyaB1 that has been used for the high-throughput screen. The histidine tag was used for affinity purification of the recombinant protein

malachite green have been reported using a microplate format (Cogan et al. 1999). In the case we describe here, the sensitivity of this method has been enhanced by the addition of pyrophosphatase to the assay, which then generates two phosphates for every cAMP molecule formed and so creates a highly sensitive adenylyl cyclase assay that should be suitable for automation.

The success of a high-throughput screen depends on several parameters that must be satisfied prior to screening. One prerequisite is a sufficient discrimination between positive and negative results. This can be assessed by determination of the Z' factor for each assay plate. The Z' parameter is based on the signal difference between positive and negative controls and the standard deviation of the controls (Zhang et al. 1999). Second, even more important than signal intensity is a high sensitivity for inhibition by chemicals in the library to be screened. In our case, inhibition of activation of PDE5-GAF-CyaB1 by cGMP is to be examined. It can be anticipated that this assay will be more sensitive for inhibition in the presence of an intermediate cGMP concentration than in the presence of a saturating cGMP concentration. As for PDE5-GAF-CyaB1, maximum activation of enzyme activity is about 45-fold and so using a cGMP concentration that causes less than half-maximal activation will still be sufficient for a high assay quality in terms of the Z' factor. Third, signal intensity should be linearly dependent on enzyme concentration and reaction time. Fourth, the assay should be validated with reference inhibitors and reveal comparable inhibition. As GAF reference inhibitors have not been described so far, GAF inhibitory compounds identified “in house” have been used as internal controls to monitor the sensitivity of our assays.

We describe here a high-throughput assay that satisfies the above parameters. The enzyme used (PDE5-GAF-CyaB1) is a recombinant chimeric adenylyl cyclase with an N-terminal hexa-histidine tag comprising the hPDE5 GAF domain (amino acids 1–513) and the catalytic adenylyl cyclase domain from CyaB1 from *Anabaena* (amino acids 386–859; gene ID 1105863, Fig. 1). Three-hundred milligrams of the recombinant protein was produced in several batches in *E. coli* as described and pooled (Bruder et al. 2006). Protein purification was optimized to achieve a high cGMP-activation factor. A phosphate calibration curve with phosphate concentrations ranging from 0 to 4 nmol phosphate/reaction tube was linear up to an OD₆₅₀ of 0.5 (Fig. 2a). The malachite green assay can detect 500 pmol phosphate in a 40 μ l sample in the presence of 75 μ M ATP, which is the planned substrate concentration. Including pyrophosphatase in the assay, 250 pmol of hydrolyzed cAMP can be detected reliably. According to our own

experimental results and previously published data (Bruder et al. 2006), the K_m of PDE5-GAF-CyaB1 for ATP is 15 μM . The assay was performed at 75 μM ATP, which is close to substrate saturation. A negative protein control was prepared from *E. coli* that was transformed with an empty vector (Fig. 2b). This protein was inactive when assayed in parallel to PDE5-GAF-CyaB1. Obviously, it had no ATPase activity, which otherwise would have released phosphate from substrate ATP. The activity of PDE5-GAF-CyaB1 was dependent on Mg^{2+} ions, and 10 mM Mg^{2+} was used in the assay. cGMP concentration/activation curves were carried out from 0 to 50 μM cGMP using reaction times of 30, 60, and 90 min (Fig. 3a). For all tested reaction times, half-maximal activation was observed with approximately 13 μM cGMP in accordance with earlier data (Bruder et al. 2006). These concentrations for half-maximal activation are significantly higher than the K_d values reported for cGMP-binding (0.027–1.9 μM), which remains an unexplained discrepancy (Liu et al. 2002; Zoraghi et al. 2005). A cGMP concentration of 10 μM cGMP was chosen for the high-throughput assay. While PDE5-GAF-CyaB1 was not enzymatically active at room temperature, the reaction rate increased in the expected fashion from 30 to 45°C, reflecting the temperature optimum of the cyclase (Kanacher et al. 2002). The assay was conducted at 37°C in order to provide a physiological temperature for the mammalian PDE5 GAF domain. Assay solutions without ATP were preincubated for 15 min at 37°C prior to substrate addition to minimize temperature changes during the reaction. Furthermore, a concentration–response curve was established

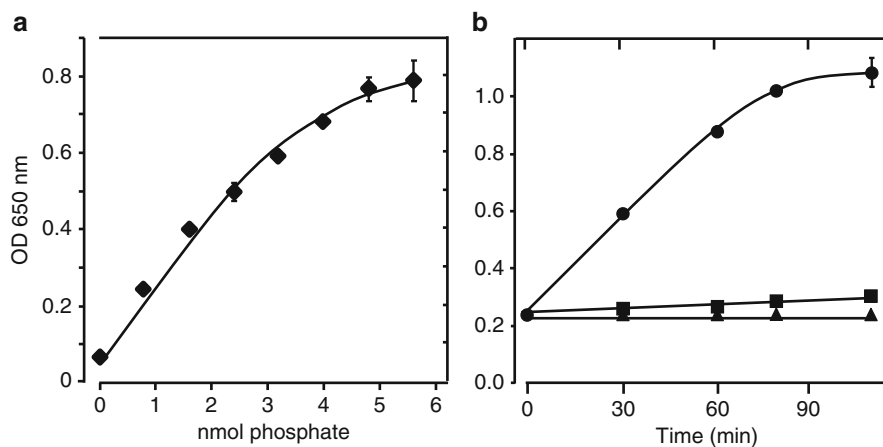


Fig. 2 Determination of adenylyl cyclase activity by measurement of released phosphate. (a) Phosphate calibration curve of the malachite green assay. 40 μl of malachite reagent was added to 40 μl of phosphate in assay buffer containing 75 μM ATP. OD_{650} was measured after 10 min using a Powerwave HT reader (Biotek). (b) PDE5-GAF-CyaB1 and a negative protein control were assayed under conditions of the malachite assay used in the high-throughput screen ($\pm 10 \mu\text{M}$ cGMP). Each point represents the average of quadruplicates. *Square* PDE5-GAF-CyaB1, *circle* PDE5-GAF-CyaB1 + 10 μM cGMP, *triangle* protein negative control $\pm 10 \mu\text{M}$ cGMP (signals \pm cGMP were identical). *Error bars* when exceeding the size of the symbol are SD ($n = 4$)

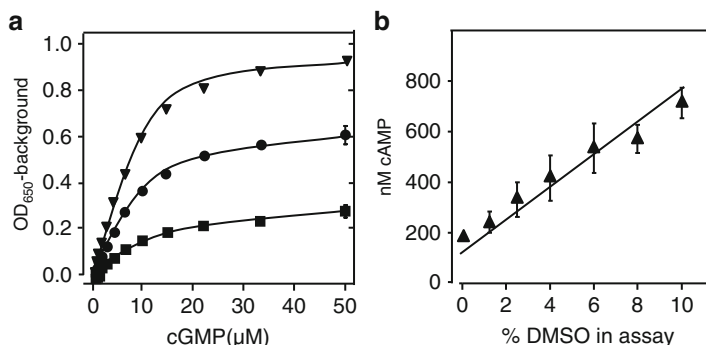


Fig. 3 Establishment of assay conditions for a high-throughput adenylate cyclase assay. (a) cGMP dependence of PDE5-GAF-CyaB1 at three different time points using the adapted malachite assay (*square* 30 min, *circle* 60 min, *inverted triangle* 90 min reaction time). (b) DMSO dependence of PDE5-GAF-CyaB1. Protein was incubated for 40 min in the presence of DMSO. The concentration of cAMP in an 8 μ l sample was determined using the cAMP dynamic 2 kit (Cisbio). Error bars when exceeding the size of the symbol are SD ($n = 4$)

for DMSO as this was used as a solvent for library compounds. Up to 10% DMSO enhanced cGMP-stimulated adenylyl cyclase activity almost linearly. At 10% DMSO, activation was fourfold (Fig. 3b). Due to the effect of DMSO on enzyme activity, minimization of the variance of automated pipetting of compounds that are dissolved in DMSO was extremely important. Pipetting variance was routinely checked and found to be 2–3%.

Based on the described features of PDE5-GAF-CyaB1, the conditions for the high-throughput assay were defined (80 min at 37°C, in the presence of 10 μ M cGMP). Under these conditions, 100 ng PDE5-GAF-CyaB1 protein in a 40 μ l sample revealed an OD₆₅₀ of 0.35 and a linear dependence of signal intensity on enzyme concentration and reaction time (not shown).

The assay principle was verified by testing the PDE5 inhibitor sildenafil. Known PDE5 inhibitors inhibit by binding to the catalytic site of PDE5. As expected, sildenafil up to 10 μ M had no effect in this assay because PDE5-GAF-CyaB1 does not contain the catalytic domain of PDE5 (an example is shown in context with Fig. 7a).

Finally, the stability of all reagents was checked. All solutions were stable for at least 24 h at 2°C and thus suitable to be used on a robotic system.

3 Running a High-Throughput Screen for the hPDE5 Tandem GAF Domain as a Drug Target

A high-throughput screen with several 100,000 compounds was carried out using white 384-well plates with clear bottom. Twenty-five percent of additional data points were spaced in between as internal controls. The assay components in 40 μ l

were 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 24 μM compound in DMSO, 2.4% DMSO, 10% glycerol, 0.005% Tween 20, 0.01% BSA, 5 mM glutathione, 75 μM ATP, 10 μM cGMP, 100 ng protein of the PDE5-GAF-CyaB1 chimera, and 0.02 unit pyrophosphatase from *E. coli*. Each plate comprised positive controls (with cGMP), negative controls (without cGMP), and background controls (without enzyme). At predetermined intervals, an additional dummy plate was assayed comprising DMSO instead of compound solutions, compounds with known effects or controls for pipetting accuracy using a fluorescent dye. Reactions were stopped after 80 min at 37°C by the addition of 40 μl of malachite reagent (8.5 mM ammonium molybdate, 0.34% malachite green, and 1 M HCl), and the OD at 650 nm was determined 10 min afterward. Compounds that absorb light of 650 nm interfere with this colorimetric assay and were excluded from evaluation. In the primary screen, the library was assayed at a single concentration. Continuous monitoring of data by the Assay Analyzer software from Genedata revealed excellent data quality with an averaged *Z'* factor of 0.75 and a 12-fold activation of the enzyme at 10 μM cGMP. As in every high-throughput screen, limited solubility of compounds can lead to reduced compound activity and false-negative results. The high-throughput screen revealed approximately 1,400 hits with an inhibition of the adenylyl cyclase reaction by >45%. These hits were subsequently retested: The assay setup will not only reveal inhibitors of the tandem GAF-mediated cGMP activation, but also inhibitors of the adenylyl cyclase reporter enzyme and compounds that interfere with protein stability. Elimination of these interferences requires additional, secondary assays.

4 Evaluation and Retest of Hits

From the 1,400 initial hits, 1,246 were retested with PDE5-GAF-CyaB1 both under the conditions of the primary screen and in additional retests without cGMP and with a saturating concentration of 100 μM cGMP. Compounds that are equally active under activated conditions (in the presence of 10 μM cGMP or more) and nonactivated conditions (without cGMP) are not considered to exert inhibition by interaction with the tandem GAF domain. The comparison of inhibition at 10 and 100 μM cGMP will reveal if compounds compete with cGMP for binding to the tandem GAF domain.

In the retest that we carried out under the conditions of the primary assay, 924 compounds revealed IC₅₀ values of more than 10 μM and were excluded from further experiments due to insufficient inhibitory potency (IC₅₀ > 10 μM). Twenty two from these were weakly active in the presence of 10 μM cGMP, but inactive in the absence of cGMP. These substances were useful in subsequent evaluations in order to define chemical clusters of compounds that bind to the GAF domain.

Three-hundred and twenty-two compounds had an IC₅₀ of 10 μM or less in the presence of 10 μM cGMP. Upon further testing, these compounds could be grouped into four categories.

The first group of 92 compounds inhibited the adenylyl cyclase reporter enzyme of PDE5-GAF-CyaB1 and had no discernible effect on GAF-mediated allosteric regulation. These compounds were equally active at saturating cGMP concentrations of 100 μM and at 10 μM cGMP and, thus, are not considered to be competitive with cGMP. This group of chemicals may be of interest as inhibitors of adenylyl cyclase in mammals because the cyanobacterial cyclase is a class III isoform as are all eukaryotic isoforms. NYC92338 (shown in Fig. 4) is a representative for the first group of compounds. In the absence of cGMP, it inhibited nearly as potent ($\log\text{IC}_{50} = -5.7$) as in the presence of 10 or 100 μM cGMP ($\log\text{IC}_{50} = -6.1$) (Fig. 5a).

The second group comprised 166 compounds that inhibited both cyclase activation via the GAF domain and the adenylyl cyclase reporter enzyme. Most of these were equally effective in the retests with 10 and 100 μM cGMP and, thus, were not competitive with respect to cGMP. The large number of compounds in this group suggests that the site of action may be a purine-binding site in either subdomain of PDE5-GAF-CyaB1. NYC292005 (shown in Fig. 4) is a representative for the second group: In the absence of cGMP a partial inhibition of PDE5-CyaB1 was observed ($\log\text{IC}_{50} = -5.4$), while in the presence of 10 μM or 100 cGMP the inhibition was complete ($\log\text{IC}_{50} = -5.6$) (Fig. 5b); thus, both the tandem GAF domain and other parts of the chimeric protein are involved in inhibition.

The third group comprised seven compounds that inhibited PDE5-GAF-CyaB1 in spite of activating the adenylyl cyclase reporter in PDE5-GAF-CyaB1. As the adenylyl cyclase activity determines signal intensity, it is assumed that these compounds inhibit GAF activation more potently than observed in this assay. NYC57569 (Fig. 4) is a representative for this group (Fig. 5d, e). These chemicals could, in principle, be of further interest as GAF inhibitors. However, due to undesirable chemical and physicochemical properties, these compounds were excluded from further evaluation.

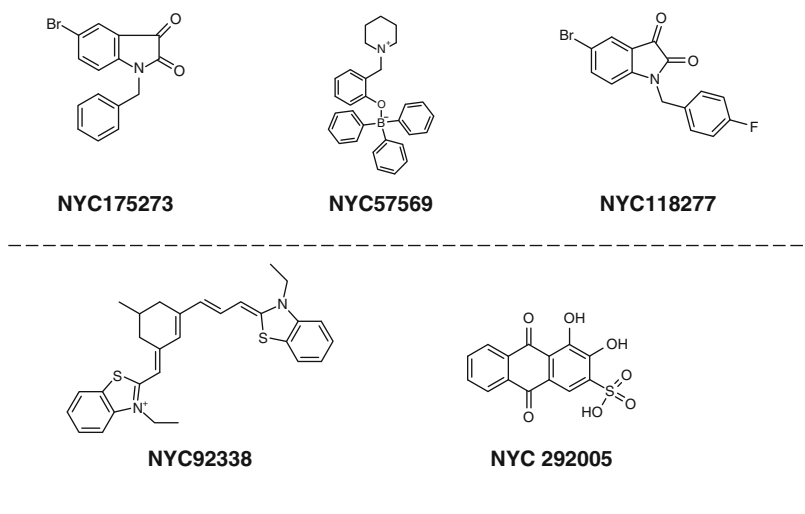


Fig. 4 Chemical structures of compounds used in retests (see above)

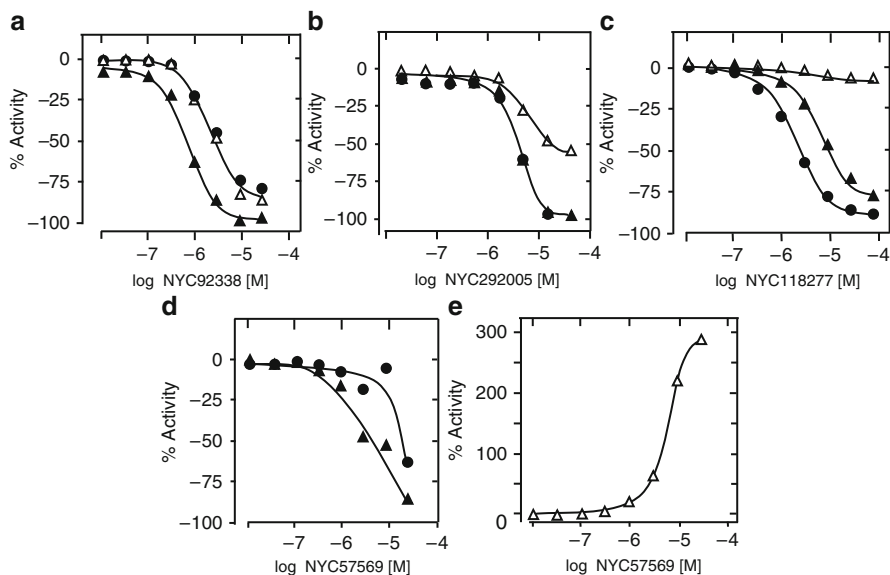


Fig. 5 Retest of compounds using the PDE5-GAF-CyaB1 malachite assay at 10 or 100 μM cGMP. (a) NYC92338 is an example for a group of compounds that inhibit mainly the adenylyl cyclase. (b) NYC292005 is an example for a group of compounds that inhibit both the adenylyl cyclase part of PDE5-GAF-CyaB1 and the GAF domain. (c) NYC118277 is an example for a group of compounds that inhibit cyclase activation via the GAF-tandem domain. (d) NYC57569 is an example for a group of compounds that inhibit the chimeric PDE5-GAF-CyaB1 protein in spite of activation of the adenylyl cyclase part of PDE5-GAF-CyaB1 shown in (e). 0% activity corresponds to the average signal obtained with a positive control. An activity of -100% corresponds to the average signal obtained with a negative control. Test conditions/symbols for a-d: closed circles 100 ng PDE5-CyaB1 protein, 10 μM cGMP, open triangles 1 μg PDE5-GAF-CyaB1, w/o cGMP, filled triangles 40 ng PDE5-CyaB1, 100 μM cGMP

The last group of 57 compounds clearly inhibited activation of the GAF domain and did not interfere with adenylyl cyclase activity. These compounds are less active in the presence of 100 μM cGMP compared to 10 μM cGMP and are, therefore, expected to compete with cGMP for binding to the GAF domain. NYC118277 (Fig. 4) is a representative for this last group: It is considered to be competitive with cGMP because it inhibited more potently at 10 μM cGMP ($\log\text{IC}_{50} = -5.8$) than at 100 μM cGMP ($\log\text{IC}_{50} = -5.2$) and not at all in the absence of cGMP (Fig. 5c).

Taken together the high-throughput screen and further testing using the PDE5-GAF-tandem-CyaB1 adenylyl cyclase chimera identified four classes of compounds that may be of future interest for different reasons. Foremost, the identification of compounds that clearly stimulate or inhibit class III adenylyl cyclases is exciting because all mammalian adenylyl cyclases are class III isoforms. It would be worthwhile to investigate whether these compounds have similar inhibitory effects on the nine isoforms of the mammalian membrane-bound adenylyl cyclases.

5 Hit Confirmation Using Assays with Human PDE5

From 1,242 retested compounds, 67 were selected for hit confirmation using hPDE5. The selection was based on chemical properties (e.g., elimination of reactive compounds), biological properties (e.g., elimination of compounds that are active on multiple targets), and physicochemical properties. Considering that the affinity of cGMP for the regulatory GAF domain is lower than for the catalytic domain (Corbin et al. 2000; Rybalkin et al. 2003b), an allosteric inhibitor of the GAF domain is expected to inhibit hPDE5 only under conditions of increased intracellular cGMP levels, while basal PDE5 activity at low cGMP concentrations should remain unaffected. On the other hand, a classical PDE5 inhibitor that competes with cGMP at the catalytic center is expected to inhibit more effectively at lower cGMP concentrations.

To compare the activity of nonactivated and activated PDE5, a two-step assay procedure has been described. Protein is preincubated with up to 100 μM of cGMP on ice to activate PDE5, rapidly diluted, and, after addition of a ^3H -cGMP spike, enzyme in 5 min at 37°C activity is determined. Under these conditions, a four- to sixfold activation by cGMP is observed (Rybalkin et al. 2003a). Using a similar, but nonradioactive assay setup, we were unable to validate this assay with in-house identified inhibitors of the tandem GAF domain in PDE5-GAF-CyaB1. Instead, we used an alternative approach: Hydrolysis of fluorescein-tagged cGMP (Molecular Devices) by native hPDE5 was detected by capillary electrophoresis using the Labchip 3000 reader (Caliper Life Sciences). The enzymatic reaction was carried out in 384 assay plates. Afterward, fluorescein-tagged cGMP and 5'-GMP were separated in a microfluidic device, the 12 sipper chip, and detected fluorimetrically after excitation with a 488 nm laser by a CCD camera combined with a 510–550 nm band pass filter (Fig. 6a). Peak heights of fluorescein-tagged cGMP and 5'-GMP are used to calculate the percent of cGMP hydrolysis. Nonfluorescent constituents are not detected and do not interfere. Fluorescein-tagged cGMP (200 nM) was used as substrate either alone or in combination with untagged cGMP in order to obtain a total cGMP concentration of either 5 or 20 μM . Control experiments demonstrated that hydrolysis of fluorescein cGMP is representative of total cGMP hydrolysis. Thus, this assay is suitable for a broad range of cGMP concentrations. According to preliminary experiments (e.g., Fig. 6b), suitable enzyme amounts for the three total cGMP concentrations were determined in order to obtain a turnover of 35% within 90 min at room temperature and a linear dependence of the turnover on enzyme amount and reaction time.

A lysate of human platelets served as source for native hPDE5 (WO 2008/095835): Platelet-rich plasma was prepared by centrifugation of citrate-treated blood ($200 \times g$, 10 min), and 1/10 volume of 85 mM Na_3 citrate/111 mM D -glucose/71 mM citric acid/pH 4.4 was added. After centrifugation ($1,400 \times g$, 10 min), the pellet was first suspended in 140 mM NaCl/3.8 mM KCl/1 mM EGTA/1 mM MgCl_2 /20 mM Tris-HCl/1 mM beta-mercaptoethanol/pH 8.2 plus a protease-inhibitor cocktail, then sonicated, and centrifuged (15 min, $10,000 \times g$).

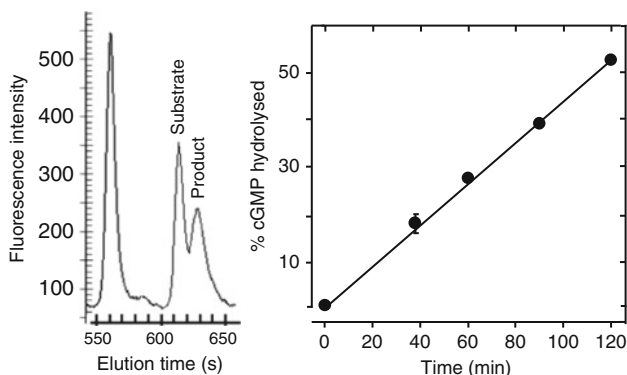


Fig. 6 Establishment of a capillary electrophoresis phosphodiesterase assay. (a) Raw data of an hPDE 5 assay with 200 nM fluorescein-tagged cGMP as a substrate as detected by capillary electrophoresis using a 12 sipper chip and the Labchip 3000 reader (Caliper Life Sciences; conditions: upstream voltage -250 V, downstream voltage $-2,500$ V, -2.7 bar). Shown is the elution profile of one sipper (two sips) with a negative control eluting between 550 and 600 s and a positive sample with cGMP and GMP eluting between 600 and 650 s. To analyze a 384-well plate, each of the 12 sippers takes in 32 sips in a defined geometric fashion. Substrate and product peaks are automatically assigned to individual wells, and percent turnover is calculated from the relative peak heights of substrate and product. (b) Time course of the hPDE5 capillary electrophoresis assay using 200 nM fluorescein-tagged cGMP as a substrate to determine a suitable enzyme amount for retesting with native PDE5 from platelet cytosol (quadruplicates). Note the excellent reproducibility of the assay as evident by the minute *error bars*. In the screen, cGMP turnover was adjusted to 35% (90 min, 20°C) by adding a suitable amount of enzyme protein

The assays were carried out on the robotic system in 384-well plates. Within $40\ \mu\text{l}$, the samples comprised 2.4% DMSO, 20 mM HEPES, 0.01% Tween 20, 0.01% BSA, 1 mM MgCl_2 , 10 μM of the PDE3 inhibitor Motapizone, 100 nM of the PDE2 inhibitor Bay 60-7550, and 0.2, 5, or 20 μM cGMP. The reaction was stopped by the robot with $40\ \mu\text{l}$ of stop buffer (100 mM HEPES, 5% DMSO, 1% coating reagent from Caliper Life Sciences, 5 mM EDTA, pH 7.4). Then the plates were transferred to the Labchip 300 capillary electrophoresis reader (Caliper Life sciences) and detection was carried out automatically overnight, thus opening up this method to medium throughput. The assay was validated by means of a concentration–response curve for the established PDE5 inhibitor sildenafil ($\log\text{IC}_{50} = -8.2$ when assayed with 200 nM fluorescein-tagged cGMP as substrate; Fig. 7c).

Sixty-seven compounds were assayed at three cGMP concentrations (see above). Fourteen compounds inhibited hPDE5 activity better or equally good at 20 μM compared to 200 nM cGMP. This indicates that these compounds inhibited both activation via GAF domain and the catalytic site of PDE5. NYC175273, an inhibitor of PDE5-GAF–CyaB1 (Fig. 7c), is shown as a representative example for this group of compounds (Fig. 7d). Two further compounds inhibited exclusively at higher cGMP concentrations. We assume that they act as allosteric inhibitors at the GAF domain, while the catalytic site of PDE5 remains unaffected.

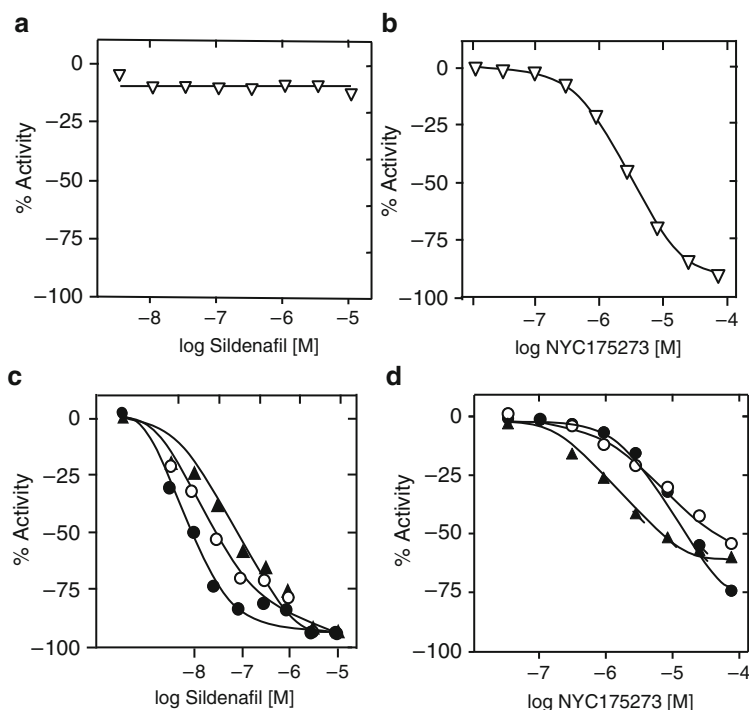


Fig. 7 Analysis of mode of action using the PDE5-GAF-CyaB1 malachite assay in presence of 10 μM cGMP and the hPDE5 capillary electrophoresis assay at different cGMP concentrations. (a) Concentration-response curve for sildenafil and (b) for NYC175273 in the PDE5-GAF-CyaB1 assay. (c) Concentration-response curves for sildenafil and (d) for NYC175273 in the hPDE5 assay. 0% activity corresponds to the average activity of the respective positive controls. -100% activity corresponds to the average activity of the respective negative controls. Assay conditions of the hPDE5 assays (c and d): filled circle with 200 nM cGMP, open circle with 5 μM cGMP, filled triangle with 20 μM cGMP. Note that sildenafil, an inhibitor of the PDE5 catalytic domain, does not inhibit the chimeric protein PDE5-GAF-CyaB1 (a). When assayed with hPDE5 (c), it is more active in the presence of a low substrate concentration (log IC_{50} = -8.2 at 200 nM cGMP; -7.6 at 5 μM cGMP; and -7.0 at 20 μM cGMP). NYC175273 is an example for a compound that inhibits the chimeric protein PDE5-GAF-CyaB1 due to interaction with the PDE5 GAF-tandem domain (b). When assayed with hPDE5 (d), it is more active under activated conditions in the presence of a high cGMP concentration (log IC_{50} = -4.7 at 200 nM cGMP; -4.7 at 5 μM cGMP; and -5.9 at 20 μM cGMP). Data evaluation of library compounds was carried out using Condoseo software from Genedata

6 Conclusions and Outlook

A sensitive colorimetric phosphate assay has been established that is suitable as high-throughput assay for the PDE5-GAF-CyaB1 fusion protein. Combined with several secondary assays including an automated capillary electrophoresis system,

we identified 16 compounds that inhibited the allosteric activation of the hPDE5 via the tandem GAF domain. Two from these inhibited hPDE5 exclusively by interaction with the tandem GAF domain while the catalytic domain was not inhibited, thus fulfilling the initial requirements. All inhibitors of the hPDE5 tandem GAF domain were assayed in additional PDE2 assays and revealed to be inhibitory under activating and nonactivating conditions (with and without the allosteric PDE2 activator cGMP), which indicates interference with the catalytic site, but not the allosteric site of PDE2 (unpublished results). Efforts have been started to crystallize the newly identified GAF inhibitors together with the hPDE5 GAF domain. Crystallization data might lead to a deeper understanding of the mechanism of binding of the hPDE5 GAF inhibitors and will help to optimize binding properties.

In addition to the GAF inhibitors, the retests with the chimeric enzyme PDE5-GAF-CyaB1 revealed another potentially highly interesting group of chemicals that inhibited the class III adenylyl cyclase reporter enzyme.

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References

- Aravind L, Ponting CP (1997) The GAF domain: an evolutionary link between diverse photo-transducing proteins. *Trends Biochem Sci* 22:458–459
- Aversa A, Bruzziches R, Pili M, Spera G (2006) Phosphodiesterase 5 inhibitors in the treatment of erectile dysfunction. *Curr Pharm Des* 12:3467–3484
- Beavo JA, Brunton LL (2002) Cyclic nucleotide research – still expanding after half a century. *Nat Rev Mol Cell Biol* 3:710–718
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Bergstrand H, Kristoffersson J, Lundquist B, Schurmann A (1977) Effects of anti-allergic agents, compound 48/80, and some reference inhibitors on the activity of partially purified human lung tissue adenosine cyclic 3', 5'-monophosphate and guanosine cyclic 3', 5'-monophosphate phosphodiesterases. *Mol Pharmacol* 13:38–43
- Bruder S, Schultz A, Schultz JE (2006) Characterization of the tandem GAF domain of human phosphodiesterase 5 using a cyanobacterial adenylyl cyclase as a reporter enzyme. *J Biol Chem* 281:19969–19976
- Chasin M, Harris DN (1976) Inhibitory and activators of cyclic nucleotide phosphodiesterase. *Adv Cyclic Nucleotide Res* 7:225–264
- Cogan EB, Birrell GB, Griffith OH (1999) A robotics-based automated assay for inorganic and organic phosphates. *Anal Biochem* 271:29–35
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- Corbin JD, Blount MA, Weeks JL 2nd, Beasley A, Kuhn KP, Ho YS, Saidi LF, Hurley JH, Kotera J, Francis SH (2003) [3H]sildenafil binding to phosphodiesterase-5 is specific, kinetically heterogeneous, and stimulated by cGMP. *Mol Pharmacol* 63:1364–1372
- Corbin JD, Turko IV, Beasley A, Francis SH (2000) Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. *Eur J Biochem* 267:2760–2767

- Croom KF, Curran MP (2008) Sildenafil: a review of its use in pulmonary arterial hypertension. *Drugs* 68:383–397
- Francis SH, Turko IV, Corbin JD (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog Nucleic Acid Res Mol Biol* 65:1–52
- Gross-Langenhoff M, Hofbauer K, Weber J, Schultz A, Schultz JE (2006) cAMP is a ligand for the tandem GAF domain of human phosphodiesterase 10 and cGMP for the tandem GAF domain of phosphodiesterase 11. *J Biol Chem* 281:2841–2846
- Handa N, Mizohata E, Kishishita S, Toyama M, Morita S, Uchikubo-Kamo T, Akasaka R, Omori K, Kotera J, Terada T (2008) Crystal structure of the GAF-B domain from human phosphodiesterase 10A complexed with its ligand, cAMP. *J Biol Chem* 283:19657–19664
- Hofbauer K, Schultz A, Schultz JE (2008) Functional chimeras of the phosphodiesterase 5 and 10 tandem GAF domains. *J Biol Chem* 283:25164–25170
- Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 35:91–100
- Houslay MD, Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J* 370:1–18
- Houslay MD, Schafer P, Zhang KY (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10:1503–1519
- Kanacher T, Schultz A, Linder JU, Schultz JE (2002) A GAF-domain-regulated adenylyl cyclase from *Anabaena* is a self-activating cAMP switch. *EMBO J* 21:3672–3680
- Liu L, Underwood T, Li H, Pamukcu R, Thompson WJ (2002) Specific cGMP binding by the cGMP binding domains of cGMP-binding cGMP specific phosphodiesterase. *Cell Signal* 14:45–51
- Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 109:366–398
- Manallack DT, Hughes RA, Thompson PE (2005) The next generation of phosphodiesterase inhibitors: structural clues to ligand and substrate selectivity of phosphodiesterases. *J Med Chem* 48:3449–3462
- Menniti FS, Faraci WS, Schmidt CJ (2006) Phosphodiesterases in the CNS: targets for drug development. *Nat Rev Drug Discov* 5:660–670
- Omori K, Kotera J (2007) Overview of PDEs and their regulation. *Circ Res* 100:309–327
- Pandit J, Forman MD, Fennell KF, Dillman KS, Menniti FS (2009) Mechanism for the allosteric regulation of phosphodiesterase 2A deduced from the X-ray structure of a near full-length construct. *Proc Natl Acad Sci USA* 106:18225–18230
- Rose GM, Hopper A, De Vivo M, Tehim A (2005) Phosphodiesterase inhibitors for cognitive enhancement. *Curr Pharm Des* 11:3329–3334
- Rotella DP (2002) Phosphodiesterase 5 inhibitors: current status and potential applications. *Nat Rev Drug Discov* 1:674–682
- Rybalkin SD, Rybalkina IG, Shimizu-Albergine M, Tang XB, Beavo JA (2003a) PDE5 is converted to an activated state upon cGMP binding to the GAF A domain. *EMBO J* 22:469–478
- Rybalkin SD, Yan C, Bornfeldt KE, Beavo JA (2003b) Cyclic GMP phosphodiesterases and regulation of smooth muscle function. *Circ Res* 93:280–291
- Sette C, Conti M (1996) Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. *J Biol Chem* 271:16526–16534
- Stroop SD, Beavo JA (1991) Structure and function studies of the cGMP-stimulated phosphodiesterase. *J Biol Chem* 266:23802–23809
- Vasta V, Beavo J (2004) Functions and pharmacological inhibitors of cyclic nucleotide phosphodiesterases. *Cell Transm* 20:3–8
- Wang H, Liu Y, Hou J, Zheng M, Robinson H, Ke H (2007) Structural insight into substrate specificity of phosphodiesterase 10. *Proc Natl Acad Sci USA* 104:5782–5787
- Zhang JH, Chung TD, Oldenburg KR (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 4:67–73

- Zhang KY, Ibrahim PN, Gillette S, Bollag G (2005) Phosphodiesterase-4 as a potential drug target. *Expert Opin Ther Targets* 9:1283–1305
- Zoraghi R, Bessay EP, Corbin JD, Francis SH (2005) Structural and functional features in human PDE5A1 regulatory domain that provide for allosteric cGMP binding, dimerization, and regulation. *J Biol Chem* 280:12051–12063
- Zoraghi R, Corbin JD, Francis SH (2004) Properties and functions of GAF domains in cyclic nucleotide phosphodiesterases and other proteins. *Mol Pharmacol* 65:267–278

Small Molecule Allosteric Modulators of Phosphodiesterase 4

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and Lance J. Stewart

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Abstract Phosphodiesterase 4 (PDE4) inhibitors have shown benefit in human clinical trials but dosing is limited by tolerability, particularly because of emesis. Novel cocrystal structures of PDE4 catalytic units with their regulatory domains together with bound inhibitors have revealed three different PDE4 conformers that can be exploited in the design of novel therapeutic agents. The first is an open conformer, which has been employed in the traditional approach to the design of competitive PDE4 inhibitors. The second is an asymmetric dimer in which a UCR2 regulatory helix from one monomer is placed in a closed conformation over the opposite active site in the PDE4 dimer (*trans*-capping). Only one active site can be closed by an inhibitor at a time with the consequence that compounds exploiting this conformer only partially inhibit PDE4 enzymatic activity while retaining

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potency in cellular and in vivo models. By placing an intrinsic ceiling on the magnitude of PDE4 inhibition, such compounds may better maintain spatial and temporal patterning of signaling in cAMP microdomains with consequent improved tolerability. The third is a symmetric PDE4 conformer in which helices from the C-terminal portion of the catalytic unit cap both active sites (*cis*-capping). We propose that dual-gating of PDE4 activity may be further fine tuned by accessory proteins that recognize open or closed conformers of PDE4 regulatory helices.

Keywords Phosphodiesterase 4 (PDE4) · PDE4 Gating · Accessory Proteins · Active Site · Asymmetric Conformer

1 Introduction

Four genes encode mammalian phosphodiesterase 4 (PDE4), all of which contain multiple upstream exons that undergo complex splicing to generate multiple PDE4 isoforms that differ in N-terminal amino acid sequence (Swinnen et al. 1989; Bolger et al. 1993; Monaco et al. 1994; Houslay and Adams 2003). Downstream exons are common to all splice isoforms and encode the PDE4 catalytic domain, which is highly conserved across the four genes (PDE4A–D). Three principle types of PDE4 splice isoforms are found. Long isoforms of the enzyme contain an N-terminal targeting sequence followed by an Upstream Conserved Regulatory domain known as UCR1, a Linker Region domain (LR1), a regulatory domain known as UCR2, another linker domain (LR2), the catalytic domain and, finally, an extreme C-terminal sequence that is unique to each PDE4 (Conti et al. 1995; Houslay 2001; Houslay et al. 2007) UCR1 contains a site for protein kinase A (PKA) phosphorylation (Sette and Conti 1996; Hoffmann et al. 1998). Short isoforms of PDE4 lack UCR1, while supershort isoforms contain an N-terminally truncated UCR2. The different splice isoforms of PDE4 are illustrated in Fig. 1.

UCR1 and UCR2 were described by Bolger et al. (1993) as upstream conserved regions in multiple amino acid sequence alignments of long transcripts encoding PDE4A–D. UCR1 and UCR2 represent ancestral domains of PDE4 as these are conserved in *Drosophila* (Davis et al. 1989) and *Caenorhabditis elegans* (Charlie et al. 2006) but not in *Dictyostelium* or yeast (Table 1). Thus, the UCR1 and UCR2 regulatory domains may have developed when eukaryotes made the evolutionary leap to multicellularity. Unlike mammals, which possess four PDE4 genes, *Drosophila* and *C. elegans* possess a single PDE4 gene (Day et al. 2005; Charlie et al. 2006). Nonetheless, the PKA phosphorylation site on UCR1 is conserved across organisms, as are important structural features of UCR2 as discussed below (Table 1). PKA phosphorylation of UCR1 increases PDE4 activity greater than two- to threefold in long isoforms from all four PDE4 subfamilies (Sette and Conti 1996; Hoffmann et al. 1998), while deletion of UCR2 can increase PDE4 activity as much as sixfold (Kovala et al. 1997), implying that it can exert an inhibitory effect on PDE4 catalytic activity. UCR1 and UCR2 have been proposed to act in concert as

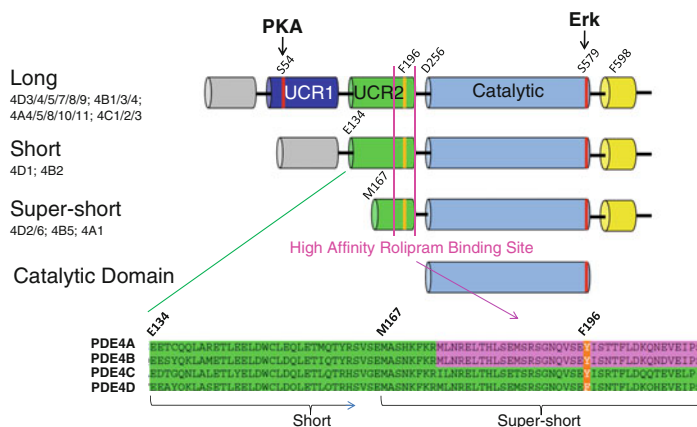


Fig. 1 Depiction of long, short and supershort splice isoforms of PDE4. Long isoforms of PDE4 contain UCR1 (blue) and UCR2 (green) regulatory domains. The catalytic domain and C-terminal sequence is identical across splice isoforms. Sites of protein kinase A (PKA) phosphorylation on UCR1 (Ser54) and Erk phosphorylation on the catalytic domain (S579) are indicated. High-affinity rolipram binding requires the C-terminal region of UCR2 (pink). UCR2 in long and short isoforms of PDE4 contains amino acids 134–222. UCR2 is truncated to amino acids 167–222 in supershort isoforms

Table 1 PDE4 UCR 2 gating elements are conserved across species

Human PDE4D	191 Asn-Gln-Val-Ser-Glu-Phe-Ile-Ser-
Asn-Thr-Phe-Leu-Asp 213	
Human PDE4B	Asn-Gln-Val-Ser-Glu-Tyr-Ile-Ser-
Asn-Thr-Phe-Leu-Asp	
<i>Drosophila</i> (SwissProt Q9W4S9)	Asn-Gln-Ile-Ser-Glu-Tyr-Ile-Cys-
Ser-Thr-Phe-Leu-Asp	
<i>C. elegans</i> (SwissProt Q22000)	Thr-Gln-Val-Ser-Lys-Phe-Leu-Ile-
Thr-Thr-Tyr-Met-Asp	
Key contact residues	----Gln-----Phe-----
-----Phe-----	

The PDE4 UCR2 α -helical gating sequence is shown for human PDE4D, Human PDE4B, *Drosophila melanogaster* (Chen et al. 1986), and *Caenorhabditis elegans* (Charlie et al. 2006). Numbering of PDE4D UCR2 is based on the reference PDE4D3 isoform (GenBank AAA97892). Gln192 is proposed to stabilize UCR2 in the closed conformation by forming a hydrogen bond with Asn528 on the surface of the PDE4 catalytic domain. The Phe196/Tyr polymorphism affects apparent K_M with respect to cAMP hydrolysis, perhaps through a hydrogen bond formed between the Tyr on UCR2 and the 2'OH of AMP or cAMP. Phe201 fits into a hydrophobic pocket on the surface of the PDE4 catalytic domain and has been shown by mutation analysis to be critical for UCR2 capping (Burgin et al. 2010)

a negative regulatory module, although structural information has been lacking (Beard et al. 2000; Houslay 2001).

PDE4 is the major cAMP hydrolyzing enzyme in most cells, tissues, and organisms. The complex pattern of splice isoforms allows PDE4 to be targeted to

different cellular microdomains and for isoform-selective regulation of PDE4 activity by accessory proteins (Houslay et al. 2007). PDE4 plays an important role in spatial and temporal patterning of signaling within cAMP microdomains (Cooper 2003; Terrin et al. 2006; Willoughby et al. 2006; Warriar et al. 2007).

2 Structure of PDE4 Regulatory Domains

The catalytic domains from seven different PDE superfamily family members (PDE1, PDE3, PDE4, PDE5, PDE7, PDE9, and PDE10) have been crystallized including all four PDE4 subtypes (4A, 4B, 4C, 4D) (Ke and Wang 2007). Comparison of these crystal structures shows that all catalytic domains have very similar overall folds, and the major features of the active site are conserved through evolution for enzymes able to hydrolyze both cAMP and cGMP to the corresponding monophosphate (Card et al. 2004). These features include two catalytic metals (magnesium and zinc) coordinated by histidine residues that activate water during catalysis (M site). In addition, nucleotide-bound structures show a conserved hydrophobic clamp (P clamp), which positions the planar purine ring of the nucleotide. Selectivity for binding adenine or guanine is not the result of different residues making differential contacts to adenine or guanine, but instead due to the conformation of a single glutamine residue (Zhang et al. 2004). The carbonyl and amine groups of the conserved glutamine make specific hydrogen bonds to the adenine or guanine base depending upon the conformation of the glutamine residue (Q switch). In some enzymes, the conformation of the glutamine residue is not constrained resulting in dual-specific enzymes able to hydrolyze both cAMP and cGMP. The understanding of the active site pocket has allowed the design of inhibitors competitive with cyclic nucleotide binding, and there are sufficient differences between different superfamily members to create family specific inhibitors (e.g., PDE4 vs. PDE5). However, the active site residues of PDE4A, 4B, 4C, and 4D are completely superimposable, and there are no sequence or structural differences that can allow the design of subtype-selective PDE4 competitive inhibitors, for example, PDE4B vs. PDE4D (Wang et al. 2007b).

We recently solved the structures of PDE4 regulatory domains with bound inhibitors (Burgin et al. 2010). Key to this achievement was cocrystallization with an atypical PDE4 inhibitor (Saldou et al. 1998). These are compounds with complex kinetics of inhibition with rolipram being the archetype (Huston et al. 1996). The bulk of industry effort has been devoted to the design of active site-directed PDE4 inhibitors with simple Michaelis–Menten kinetics that bind to the catalytic site and thus are competitive with the cAMP substrate (Wang et al. 2007b). Atypical PDE4 inhibitors such as rolipram were recognized to bind both high- and low-affinity sites on PDE4, although the structural basis of those sites was not known (Souness and Rao 1997). Clues to the basis of high-affinity rolipram binding could be gleaned from two different studies where a portion of the regulatory UCR2 domain was implicated in the generation of high-affinity binding of rolipram.

Jacobitz implicated this region by truncation analysis of PDE4A to UCR2 residues 265–332 (Jacobitz et al. 1996), Fig. 1, while Rocque and colleagues mapped a requirement for high-affinity rolipram binding to a similar region within the UCR2 of PDE4B2, namely residues 81–152 (Rocque et al. 1997). Both groups recognized that high-affinity rolipram binding required the presence of the catalytic domain and that further truncation of the enzyme to remove UCR2 yielded proteins with low-affinity rolipram binding to the catalytic site. Two additional atypical PDE4 inhibitors, RS25344 and PMNPQ, were described by Saldou, who also showed by truncation analysis a requirement for UCR2 to achieve potent enzyme inhibition (Saldou et al. 1998; Brideau et al. 1999). Whether the high-affinity rolipram binding site was an allosteric site or instead altered the conformation of the catalytic site to induce a change from low- to high-affinity rolipram binding was unclear (Jacobitz et al. 1996; Houslay 2001; Liu et al. 2001; Houslay et al. 2005).

We initially attempted cocrystallization of a truncated form of PDE4 containing the catalytic domain and C-terminal sequences. Crystal structures obtained with (R/S)-rolipram (PDB ID: 3G4K), RS25344 (PDB ID: 3G4I), and PMNPQ (PDB ID: 3G58) revealed a novel binding mode in which chemical elements determining potency were oriented out of the active site toward solvent. The three inhibitors have a central planar ring that stacks between Phe372 and Ile336, and each forms a hydrogen bond with Gln535. These features are common to all known PDE4 inhibitors (Wang et al. 2007b). Active site-directed, competitive PDE4 inhibitors often coordinate to the catalytic metals in the active site. The atypical PDE4 inhibitors, in contrast, did not contact the metals, but instead had two aromatic groups that reached out of the active site and interacted with the solvent (Fig. 2). This result was surprising since previous studies had demonstrated that modifications of either aromatic group could significantly alter the ability to inhibit long forms of PDE4 (Wilhelm and Axt 1995). Close examination of the catalytic domain also showed a groove across the active site that could, potentially, “dock” with a regulatory domain such as UCR2.

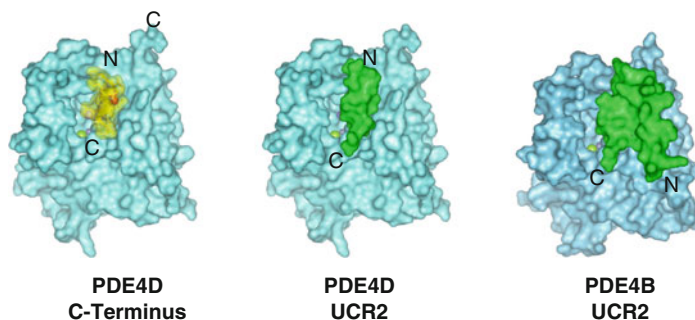


Fig. 2 Surface rendered view of PDE4 regulatory helices capping the active site. The surface rendered catalytic domain is shown in *blue*, the C-terminal helix is shown in *yellow*, and UCR2 is in *green*. The entire supershort UCR2 was visible in the PDE4B structure, while only the UCR2 gating helix is visible in the PDE4D structure (Burgin et al. 2010)

Based on these crystal structures, we surmised that a regulatory helix, most likely in UCR2, might close across the active site and contact the aromatic arms of the atypical PDE4 inhibitors. This proved to be correct as we were able to obtain cocrystal structures of RS25344 (PDB ID: 3G4G) and PMNPQ (PDB ID: 3G45) in contact with an α -helical region of UCR2 that closed across the active site (Fig. 2). The protein constructs used for cocrystallization experiments were highly engineered using our Gene Composer™ software (Lorimer et al. 2009). We modified surface residues on the catalytic domain to promote crystal packing, evaluated the effect of different linker sequences between UCR2 and the catalytic domain on crystallization, and evaluated literally hundreds of different protein constructs. Gene Composer™ also allowed us to design synthetic genes with codon usage optimized for either bacteria, insect or mammalian expression systems (Raymond et al. 2009). Indeed, we explored upward of 180 synthetic gene constructs before obtaining cocrystals of RS25344 or PMNPQ with forms of PDE4D and PDE4B containing UCR2 expressed and purified from insect cells. We found in these experiments that crystallization was ligand dependent.

Our PDE4B UCR2 cocrystal structure with PMNPQ reveals nearly a complete structure of the truncated UCR2 seen in supershort PDE4 isoforms (Fig. 2). In this, an N-terminal α -helix of UCR2 lies over one wall of the PDE4B catalytic domain. This is joined by a sharp beta-turn to a second α -helix that caps the active site and contacts the two aromatic arms of the otherwise buried PMNPQ. In the PDE4D UCR2 cocrystal structure, although the first 27 residues are disordered and not visible in the crystal lattice, a well-defined helix spanning residues Asn191 to Asp203 was observed over the active site. Unfortunately, in both structures, the LR2 linker region between UCR2 and the N-terminal portion of the catalytic domain is disordered and cannot be modeled. It is therefore not possible to unequivocally determine from these structures whether the UCR2-catalytic domain interaction is intramolecular or occurs between two different molecules in a dimer form, for example.

The UCR2 α -helix closing across the PDE4 active site is identical in sequence between isoforms, except for one key residue which contacts the PDE4 inhibitor (Table 1). This residue is a phenylalanine (Phe) in PDE4D and a tyrosine (Tyr) in PDE4A–C. Fascinatingly, the phenylalanine polymorphism in PDE4D arose in the mammalian lineage giving rise to primates, as PDE4D contains the ancestral tyrosine in mice, rats, and dogs. This then has important consequences for drug discovery when trying to extrapolate the pharmacology and tolerability of atypical PDE4 selective inhibitors from, as is invariably done, rodents to man.

In addition to the interaction with the bound inhibitor, it is important to note that closure of the UCR2 helix across the active site is stabilized by direct interactions between the helix and the PDE4 catalytic domain. For example, there is good shape complementarity between the UCR2 helix and the catalytic domain groove, Gln192 is positioned to make a hydrogen bond to the main chain carbonyl of Asn528, and Phe201 fits within a hydrophobic cleft made by Ile376 and Met439 (numbering based on PDE4D3 isoform, GenBank accession No. AAA97892). Mutation of Phe201 alters inhibitory potency of atypical inhibitors interacting with UCR2

even though these compounds do not directly contact Phe201. Gln192 and Phe201 in PDE4D UCR2 are conserved across PDE4A-C, and indeed, in UCR2 from *Drosophila* (Table 1). Despite the amino acid sequence conservation between human and *Drosophila* UCR2, *Drosophila* PDE4 is not inhibited by rolipram (Henkel-Tiggens and Davis 1990). The basis for this has yet to be resolved, although it is unlikely to be a simple Phe-Tyr switch. *C. elegans* UCR2 has a conservative substitution of Tyr at the hydrophobic docking residue equivalent to Phe201.

The UCR2 gating helix also affects the apparent K_M for cAMP. Human PDE4D has a lower K_M for cAMP (1.5 μM) as compared to PDE4B (7.7 μM). Docking studies suggest that closure of UCR2 over the active site may allow Tyr275 in PDE4B to form a hydrogen bond to the 2'OH of cAMP or 5' AMP, and indeed mutation of PDE4D Phe196 to Tyr raises the apparent K_M for cAMP to 7.5 μM as predicted (Burgin et al. 2010). Formation of a hydrogen bond between UCR2 and the 2'OH of 5' AMP may affect K_M by stabilizing the product bound form of the enzyme-substrate complex. *C. elegans* UCR2 also contains a Phe at the homologous position rather than a Tyr as in *Drosophila*, suggesting that there may have been natural selection in *C. elegans* for a low K_M PDE4.

In addition to the UCR2 regulatory helix, we solved a cocrystal structure of PMNPQ with a C-terminal α -helix closing across the active site (PDB ID: 3 G58), Fig. 2. Previous X-ray structures of PDE4B (1F0J and 1XM6) (Xu et al. 2000; Card et al. 2004), a recently deposited structure (Kranz et al. 2009), and a deposited but unpublished structure (PDB ID: 3KKT), all show a C-terminal helix spanning residues Asn422 to Phe434. C-terminal residues of PDE4 are important for protein expression and therefore present in most N-terminally truncated constructs used to crystallize the catalytic domains of PDE4B and PDE4D; however, these residues are typically disordered in the crystal lattice and are therefore absent in most models. The helices from the 1F0J and 1XM6 structures do not overlay and do not fit into the same groove that we have observed with the UCR2 helix; however, we speculated that this C-terminal helix could also come across and be visualized if stabilized by an appropriate inhibitor. In our cocrystal structure with PMNPQ, the PDE4D C-terminal helix was clearly defined in only one of the four monomers in the asymmetric unit suggesting that the inhibitor only weakly interacts with this C-terminal helix. The observed C-terminal helix does overlay with the C-terminal helix modeled in 3KKT, and with the UCR2 helix in both PDE4D and PDE4B (RMSD values of 0.90 and 0.76 Å for α -carbons) structures, and a conserved phenylalanine protruded into the active site and stacked upon the aromatic group of PMNPQ. The helix was also stabilized by a hydrophobic interaction between the helix (Leu436) and the catalytic domain (Ile376/Met439); however, we do not identify any potential hydrogen bond interactions between the helix and the catalytic domain as for UCR2. The linker between the C-terminus of the catalytic domain and the N-terminus of the helix were not visualized in the 1F0J and 1XM6 structures, and could not be modeled in our structure; however, the linker region is visible in the 3KKT structure showing an intramolecular interaction. Unlike LR2 between UCR2 and the catalytic domain, which is relatively long (>40 amino acids), the linking portion between the catalytic domain and the

C-terminal helix is short (~15 amino acids) and likely only allows the C-terminal helix to interact intramolecularly with the adjacent active site of the same monomer in all of these structures.

The UCR2 and C-terminal regulatory domain structures suggest a “dual-gating” mechanism regulating PDE4 hydrolysis of cAMP (Houslay and Adams 2010; Burgin et al. 2010). In this model, UCR2 and the C-terminal gating helix independently regulate PDE enzymatic activity (Fig. 3). The C-terminal helix likely acts intramolecularly to regulate the adjacent active site, while UCR2 may act intermolecularly. We propose that the kinetic behavior of atypical PDE4 inhibitors indicates that the two active sites are negatively cooperative such that when the UCR2 from one of the monomers in the dimer closes over the active site of the other monomer in the dimer, this engenders a greatly reduced affinity of the other UCR2 for interacting with the other active site in the proposed dimeric unit. Indeed, this can be best explained by a model in which biological isoforms of PDE4 are functional dimers (Lee et al. 2002; Richter and Conti 2002). Closing UCR2 creates an asymmetric dimer in which one active site is fully inhibited and the opposite active site has reduced catalytic activity (Burgin et al. 2010). In this model, we have suggested that UCR2 likely acts intermolecularly such that UCR2 from one monomer closes across the active site of the opposite monomer, Fig. 3 (Burgin et al. 2010). This highlights the functional importance of PDE4 dimerization with respect to regulation of activity. Indeed, accessory proteins that interact with the PDE4 monomer and thereby prevent dimerization may prevent UCR2 gating.

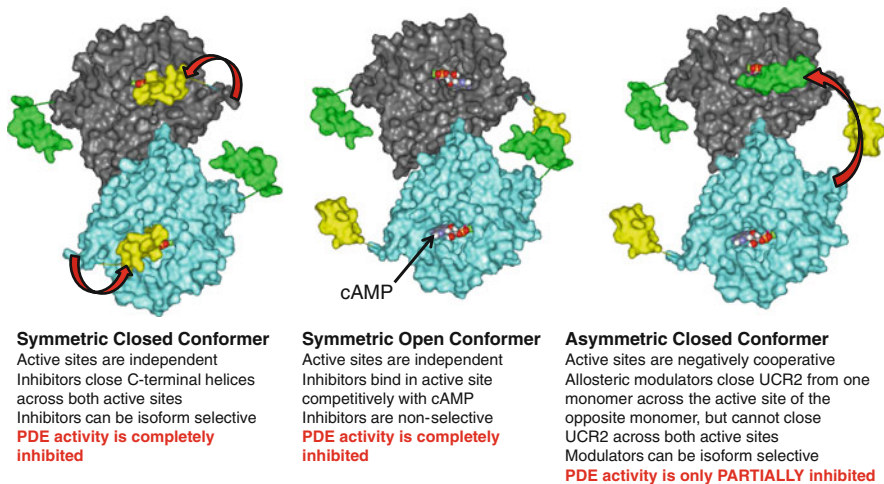


Fig. 3 PDE4 conformers that may be exploited by therapeutic agents. In the model, the PDE4 dimer can exist as a symmetric closed conformer with the C-terminal helix capping both active sites, it can exist as an open symmetric monomer with both active sites uncapped, and it can exist as an asymmetric dimer with UCR2 capping one active site, while the other active site remains open. The catalytic domains of the PDE4 monomers are surface rendered in *blue* or *magenta*. The C-terminal gating helix is rendered in *yellow*, while the UCR2-gating helix is rendered in *green* or *red*

3 Fine Tuning of PDE4 Gating by Accessory Proteins

In addition to the A Kinase Anchoring Proteins (AKAPs) which provide a scaffold for organizing PDE4 within the cell, other accessory proteins regulate PDE4 activity by interacting with PDE4 regulatory domains (Houslay et al. 2007). Examples of both positive and negative regulators of PDE4 activity have been described (Table 2). These types of accessory proteins have been shown by mutational and biochemical analysis to interact with UCR2 or with the C-terminal regulatory helix. The ability of accessory proteins to stabilize particular PDE4 conformations may be reflected in the tissue-specific distribution of high-affinity rolipram binding (Zhao et al. 2003).

Myomegalin was identified as a PDE4-interacting protein by Verde et al. (2001). Myomegalin has multiple tissue variants and can be best viewed as a scaffolding protein associated with multiple subcellular organelles including golgi, centrosomes, and skeletal muscle sarcomeres. Myomegalin interacts with long and short forms of PDE4D (PDE4D3 and PDE4D1, respectively), but not with a supershort form (PDE4D2) containing a truncated UCR2 (see Fig. 1). More importantly, PDE4D retains enzymatic activity when co-immunoprecipitated with myomegalin, suggesting that myomegalin interacting with the N-terminus of UCR2 may prevent UCR2 from gating PDE4 activity by closing across the active site. This would be predicted to impair high-affinity binding of atypical PDE4 inhibitors interacting with UCR2 in the closed conformation. As myomegalin is highly expressed in heart, such an accessory protein interaction with PDE4 may decrease the potential for cardiotoxicity by atypical PDE4 inhibitors (Lehnart et al. 2005).

Correspondingly, accessory proteins have been described, which likely recognize UCR2 in the closed conformation, thereby increasing sensitivity to atypical PDE4 inhibitors, such as rolipram (Bolger et al. 2003b). PDE4A5 was identified as a potential interactor with the immunophilin AIP/XAP2/ARA9 in a yeast two-hybrid screen (Bolger et al. 2003a). The arylhydrocarbon interacting protein (AIP) interacts with the aryl hydrocarbon receptor, which is needed for transcriptional responses to xenobiotics and for normal cardiac development (Houslay et al. 2007). AIP binding to UCR2 inhibits PDE4A5 hydrolysis of cAMP >50% in a noncompetitive fashion and increases the potency of inhibition by rolipram. In our model, rolipram may stabilize the protein interaction surface for AIP binding by shifting the equilibrium of PDE4 from an open to a closed conformation of UCR2. Accessory proteins binding the LR2 linker region between UCR2 and the catalytic domain, as shown for the SH3 domains of SRC family tyrosyl kinases in binding to the proline- and arginine-rich LR2 that is unique to PDE4A (McPhee et al. 1999), also markedly potentiate rolipram binding and elicit kinetics of partial competitive inhibition, which would be explained by our model (Burgin et al. 2010) if this interaction acted in stabilizing the closed conformation of UCR2.

DISC1 (disrupted in schizophrenia) also may hold UCR2 in a closed form, thereby inhibiting the PDE catalytic activity of the complex. Disruption of DISC1

Table 2 PDE4 accessory proteins and their effect on PDE4 localization, enzymatic activity and sensitivity to inhibition by rolipram

Accessory protein	Site on PDE4	PDE4 isoforms	Effect	Rolipram sensitivity	References
AIP/XAP2/ARA9	N-terminus and UCR2	4A4/5, 4D4	Inhibits PDE4 activity	Increased	Bolger et al. (2003b)
AKAP149	unknown	4A	Anchoring to mitochondria		Asirvatham et al. (2004)
AKAP18	N-terminus	4D3/9	Anchoring to water channel aquaporin-2-bearing vesicles		Stefan et al. (2007)
AKAP9	N-terminus	4D3, not 4D5	Anchoring to cardiac I(Ks) potassium channel		Terrenoire et al. (2009)
AKAP95	unknown	4A	Anchoring to nucleus		Asirvatham et al. (2004)
β -arrestin	N-terminus and catalytic domain	4D5, not 4D3	Dynamic recruitment to β_2 -adrenergic receptor, prevents ERK1 phosphorylation	unknown	Bolger et al. (2003a, 2006), Baillie et al. (2007)
DISC1	UCR2		Inhibits PDE4 activity	unknown	Murdoch et al. (2007)
ERK1	Catalytic domain	All	Inhibits activity of long forms of PDE4	unknown	Hoffmann et al. (1999), MacKenzie et al. (2000)
Lyn, Fyn, Src	N-terminus, LR2 linker	4A4/5, 4D4	Anchoring to SH3-domain kinases	Increased	McPhee et al. (1999)
mAKAP	N-terminus	4D3	Anchoring to cardiomyocyte perinuclear region		Carlisle Michel et al. (2004)
MTG	unknown	4A	AKAP anchoring to golgi		Asirvatham et al. (2004)
myomegalin	UCR2		Activates PDE4 activity	Decreased?	Verde et al. (2001)
PKA	UCR1	long forms	Activates PDE4 activity		Sette and Conti (1996), Hoffmann et al. (1998)
RACK1	N-terminus, C-terminal helix	4D5	Anchoring to WD repeat proteins		Yarwood et al. (1999), Bolger et al. (2006)
RyR2 ryanodine receptor	N-terminus	4D3, not 4D5	Anchoring		Lehnart et al. (2005)

by a balanced chromosomal translocation is associated in a large Scottish family with major psychiatric illness including schizophrenia, bipolar disorder, and severe recurrent depression (Blackwood and Muir 2004). DISC1 mutant mice display altered behavioral phenotypes suggestive of PDE4 dysregulation (Clapcote et al. 2007). DISC1 has been shown to interact with residues on the catalytic domain of PDE4D, and with regions of UCR2 upstream of the gating helix (Murdoch et al. 2007). This has led us to the hypothesis that DISC1 binding stabilizes the closed conformation, by simultaneously binding to both UCR2 and the catalytic domain. Consistent with this proposal, Millar et al. have shown that PKA phosphorylation disrupts DISC1 binding to PDE4B, which results in an activation of enzyme activity (Millar et al. 2005). This activation may occur as a response to increased cAMP concentration, providing an additional level of local control of PDE4 activity, in this example, perhaps at individual neuronal synapses.

Finally, the signaling scaffold protein RACK1 (receptors for activated C-kinase) has been shown to bind the N-terminal region of PDE4D5 as well as the C-terminus (Yarwood et al. 1999). The residues important for the binding of RACK1 to the C-terminus of PDE4D5 were mapped by peptide array scanning to the C-terminal regulatory helix and the face of the PDE4D catalytic domain (Bolger et al. 2006). PDE4D5 complexes with RACK1 showed no change in K_M or V_{max} toward cAMP (Yarwood et al. 1999) suggesting that RACK1 may hold the C-terminal regulatory helix in an open conformation. RACK1 may also hinder UCR2 closure, as binding of RACK1 slightly attenuates the sensitivity of PDE4D5 to inhibition by rolipram (Yarwood et al. 1999).

4 Active Site-Directed Competitive Inhibitors Bind an Open PDE4 Conformer

We suggest that the two active sites in the PDE4 dimer act independently when UCR2 and the C-terminal regulatory helix are in the open conformation (Fig. 3). As a consequence, active site-directed PDE4 inhibitors that purely compete with cAMP at the active site display simple Michaelis–Menten kinetics of enzyme inhibition. The design of competitive PDE4 inhibitors has converged on compounds that exploit a binding pose similar to cAMP (Fig. 4). Competitive PDE4 inhibitors generally contain three binding elements, which mimic those found in the cyclic nucleotide substrate. These are a hydrogen bond acceptor, a planar aromatic ring system, and functional groups that coordinate with catalytic site metals (magnesium or zinc). All PDE inhibitors have a central planar ring that stacks in a clamp (P clamp) formed by highly conserved hydrophobic residues that sandwich the inhibitor in the active site (Card et al. 2004). All PDE inhibitors also hydrogen bond to an invariant glutamine (Q switch) at the bottom of the hydrophobic clamp. In PDE4D, Phe538 and Ile502 form the walls of the hydrophobic clamp with Gln535 corresponding to the invariant glutamine. These features of the active site are

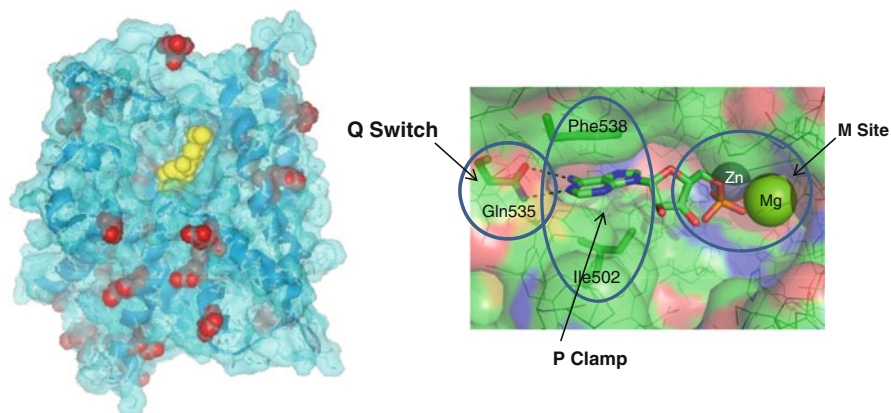


Fig. 4 Architecture of the PDE4 catalytic domain and active site. (*Left*) Surface-rendered view of the PDE4D catalytic domain (*blue*) with roflumilast (*yellow*) bound in the active site. Amino acids that differ between PDE4D and PDE4B are colored *red*. There are no sequence differences within the active site that can be used to achieve subtype selectivity. (*Right*) Surface-rendered view of the PDE4 catalytic site. The invariant glutamine (Gln535), hydrophobic P-clamp (Ile502 and Phe538), and the M-site comprising the catalytic metals are indicated

present in all PDEs. Surrounding residues anchor the glutamine in orientations that allow hydrogen binding to a ring nitrogen of cAMP or cGMP. Competitive PDE4 inhibitors have also been optimized for interaction with binding pockets in the PDE4 active site or for interaction with the catalytic metals (Wang et al. 2007a). The absolute sequence conservation of the PDE4 active site across the PDE4A–D subfamilies has made it difficult to develop subtype selective PDE4 inhibitors (Fig. 4). Indeed, potent compounds such as roflumilast are equipotent against PDE4A–D (Fig. 5).

Cilomilast and roflumilast are the competitive PDE4 inhibitors for which the most extensive clinical data are available. The compounds were developed for treating respiratory disease with a particular focus on asthma and chronic obstructive pulmonary disorder. Both compounds show noninferiority to inhaled steroids in mild-to-moderate asthma supporting the therapeutic concept of PDE4 inhibition (Reid 2002; Giembycz 2005, 2006; Lipworth 2005; Spina 2008). Key features of asthma are the recruitment of inflammatory cells to lung and airway hypersensitivity, both of which are blunted by PDE4 inhibitors. Eosinophils are sensitive to inhibition of PDE4D (Chambers et al. 2006), while inflammatory activation of monocytes is sensitive to PDE4B inhibition (Jin and Conti 2002; Spina 2008). Measurement of eosinophil or monocyte activation in human whole blood thus provides a convenient pharmacodynamic marker of PDE4 inhibition and a metric by which to evaluate therapeutic margin as shown in Table 3. Comparing the two compounds, roflumilast is the more potent PDE4 inhibitor with an *in vitro* IC₅₀ of 2 ng/ml for inhibition of eosinophil production of leukotriene E₄ (LTE₄). Monocytes are tenfold less sensitive to roflumilast than eosinophils with an

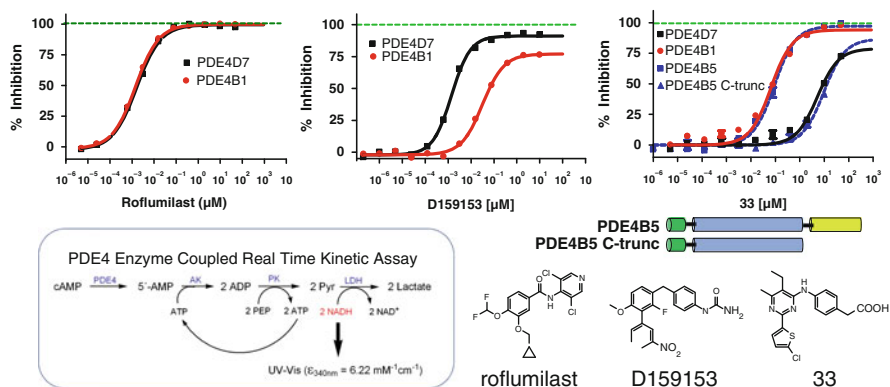


Fig. 5 Inhibition of PDE4 enzymatic activity by compounds exploiting ligand-binding modes unique to the three PDE4 conformers. Roflumilast binds the open PDE4 conformer competitively with cAMP, inhibits the enzyme completely ($I_{\max} = 100\%$), and has no subtype-selectivity for PDE4D versus PDE4B. D159153 binds the asymmetric PDE4 conformer noncompetitively with cAMP, there is a ceiling to the maximum inhibition of PDE4D ($I_{\max} = 90\%$), and the compound shows subtype selectivity due to the Phe/Tyr polymorphism in UCR2. Compound 33 (Naganuma et al. 2009) may bind the symmetric closed PDE4 conformer with the C-terminal helices capping both active sites. Compound 33 inhibits PDE4 completely ($I_{\max} = 100\%$) and has subtype-selectivity for PDE4B over PDE4D. Potency and PDE4B selectivity require the C-terminus. It inhibits long (PDE4B1) and supershort (PDE4B5) subtypes of PDE4B with equal potency. Truncation of the PDE4B5 C-terminus causes a 150-fold drop in potency from an $IC_{50} = 0.09 \mu\text{M}$ against PDE4B5 to an $IC_{50} = 14 \mu\text{M}$ when the C-terminus is removed. D159153 and compound 33 share a common chemotype characterized by a planar, aromatic ring from which a pair of aromatic arms project. Biochemical assay data were generated using a novel, real-time kinetic assay (Box) in which hydrolysis of cAMP is coupled to NADH formation, which can be measured spectroscopically (Burgin et al. 2010)

in vitro IC_{30} of 20 ng/ml with respect to inhibition of lipopolysaccharide-stimulated production of tumor necrosis factor alpha ($TNF\alpha$) (Hatzelmann and Schudt 2001). The clinical dose of roflumilast explored in human clinical trials (0.5 mg once daily) exceeded the no observable effect level (NOEL) for emesis (Table 3). Roflumilast was emetic in 8% of subjects at that dose. Emesis in animals occurs quickly and violently after dosing with such a PDE4 inhibitor, suggesting that emetic threshold is driven by the rising or maximum concentration reached by the drug. The clinical dose of roflumilast achieved a C_{\max} of 3.9 ng/ml in plasma and a steady-state average concentration (C_{av}) of 1.5 ng/ml. Thus, the clinical dose of roflumilast did not produce sustained blood levels greater than the IC_{50} for in vitro inhibition of eosinophil and monocyte inflammatory responses. Presumably, additional therapeutic benefit would be obtained if the dose could be increased, but dose escalation is limited by tolerability. Emesis can be reduced to some extent by designing PDE4 inhibitors that distribute poorly to the brain (Aoki et al. 2001), but the trade-off may be high plasma protein binding, poor cellular permeability, or low oral bioavailability.

Table 3 Effective inhibition of PDE4 subtypes achieved in human clinical trials related to emesis

	Units	Cilomilast	Roflumilast	Roflumilast N-oxide
MW	gm/mol	343	403	419
hPDE4D7 IC ₅₀	ng/ml	36.7	1.7	4.6
hPDE4B1 IC ₅₀	ng/ml	122	2.9	4.3
LTE4 IC ₅₀	ng/ml	395	2.2	13.4
TNF α IC ₃₀	ng/ml	1,715.0	20.2	21.0
Clinical dose		15 mg b.i.d.	0.5 mg q.d.	
C _{max}	ng/ml	990	3.9	8.4
AUC (0-24)	ng.h/ml	22,000	35	147
C _{av} (AUC/24)	ng/ml	916.7	1.5	6.1
Occurrence of adverse events		>10% Subjects reported nausea	Diarrhea (8%), nausea (8%)	
Therapeutic index	Clinical C _{max} / LTE4 IC ₅₀	2.5	1.7	0.6
LTE4 effective dose (C _{av})	% Inhibition	ED70	ED39	ED31
TNF α effective dose (C _{av})	% Inhibition	ED19	ED3	ED11/ED22(ss)

Biochemical in vitro assay data (hPDE4D7, hPDE4B1) and human whole blood LTE4 production after Sephadex-stimulation (LTE4 IC₅₀) are from [(Burgin et al. 2010) and unpublished]; data on human whole blood TNF α production after LPS-stimulation are from (Hatzelmann and Schudt 2001). Cilomilast clinical dose, pharmacokinetic data and occurrence of adverse events are from (Giembycz 2006) or from US FDA Docket 3976B1, Pulmonary-Allergy Drugs Advisory Committee, Preclinical Considerations, FDA, Table 1; single dose AUC. Roflumilast clinical dose, pharmacokinetic data and occurrence of adverse events are from (Lipworth 2005) or Wurz W (2002) Altana Pharma, Roflumilast, Frankfurt/Main slides 21-22.

5 UCR2-Directed Allosteric Modulators Bind an Asymmetric PDE4 Conformer

The binding pose of UCR2-directed allosteric modulators exploits an asymmetric PDE4 conformer in which one active site is capped by UCR2, while the second active site remains open (Fig. 3). Polymorphism in a key residue on UCR2, Phe196 in PDE4D and Tyr274 in PDE4B, has allowed the design of compounds that are 100-fold selective for either PDE4D (Burgin et al. 2010) or PDE4B (unpublished). Compounds acting allosterically to close UCR2 act as bi-dentate ligands with one end binding UCR2 and the other binding within the active site.

Atypical PDE4 inhibitors such as rolipram, RS25344, and PMNPQ that bind to UCR2 (Jacobitz et al. 1996; Rocque et al. 1997; Saldou et al. 1998; Burgin et al. 2010) are highly emetic (Hebenstreit et al. 1989; Robichaud et al. 1999, 2001). However, early in our studies of the structure–activity relationship (SAR) for compounds binding to UCR2, we identified compounds that did not completely inhibit the enzymatic activity of the PDE4 dimer due to negative cooperativity between the two active sites. I_{\max} was a maximum of 85–90%. I_{\max} was decreased

more than 50% for the dimer as closing UCR2 across one active site also reduced the k_{cat} of the other active site (Burgin et al. 2010). We thought such partial inhibitors might provide a “safety valve” preventing complete enzyme inhibition and thereby improve tolerability.

Compounds able to bind UCR2 when located in the active site share a common pharmacophore and binding pose. The pharmacophore consists of four elements; a planar scaffold providing a hydrogen bond to Gln535, a linker, and two aromatic substituents which create a clamp that holds UCR2 in the closed conformation. Key to the design of partial inhibitors was the deliberate weakening of the P-clamp interaction with the hydrophobic walls of the active site. For example, we obtained potent compounds based on pyridine and quinoline scaffolds, but compounds based on bicyclic ring systems fully inhibited PDE4 activity. In contrast, compounds designed with catechol and biaryl scaffolds having less aromaticity afforded compounds with both full and partial inhibition behavior. With respect to the aromatic arms, subtle changes to Ar1 substituents affected full or partial inhibition behavior. Flexibility in Ar1 may allow full inhibitors to access additional interactions with active site residues. Ar2 substituents affected potency but not full or partial kinetic behavior. Full and partial inhibitors close UCR2 across the active site identically, so differences in the positioning of UCR2 do not explain the differences in kinetic behavior (Burgin et al. 2010).

We found that examples of PDE4 allosteric modulators were potent in cellular assays. Studies of eosinophil production of LTE4 in human whole blood demonstrated that PDE4 allosteric modulators could provide complete inhibition of a biological response, even though they have partial enzyme inhibition kinetics in biochemical assays (Burgin et al. 2010). For reference, simple competitive PDE4 inhibitors, such as roflumilast, exhibit similar potency in biochemical assays, by assay of inhibition of cAMP hydrolysis in forskolin-stimulated HEK293 cells, and in an eosinophil assay of LTE4 production (IC_{50} values were 5.8 nM, 4 nM and 5.6 nM, respectively, across the three assays). PDE4D is the dominant PDE4 subtype in human embryonic kidney HEK293 cells with respect to cAMP hydrolysis (Lynch et al. 2005). In contrast to roflumilast, PDE4 allosteric modulators showed similar potency with respect to inhibition of PDE4D in biochemical assays and in the eosinophil assay, but were 10- to 15-fold less potent with respect to inhibition of cAMP hydrolysis in HEK293 cells. For example, the clinical lead compound D159687 had IC_{50} of 28 nM and 44 nM, respectively, for biochemical inhibition of PDE4D and inhibition of eosinophil LTE4 production, but an IC_{50} of 253 nM with respect to inhibition of cAMP hydrolysis in HEK293 cells (Burgin et al. 2010). Rolipram similarly is potent in biochemical and eosinophil assays but tenfold less potent versus inhibition of cAMP hydrolysis. Why should this be so?

Differences between membrane-bound and cytosolic PDE4 with respect to inhibition by rolipram have been noted previously (Huston et al. 1996; Souness and Rao 1997). Huston et al. report that rolipram inhibits membrane-bound PDE4 with high affinity, suggesting that the compound is inhibiting PDE4 through closing UCR2. Rolipram was found to be much less potent against cytosolic PDE4, which behaved kinetically as if rolipram only competed with cAMP for binding

in the active site (Huston et al. 1996). Imaging of cAMP concentrations in cortical neurons using biosensors also suggests that submembrane and cytosolic cAMP microdomains are affected differently by rolipram (Castro et al. 2010). Rolipram affects free cAMP more strongly in the apical dendrites of parietal cortex neurons as compared to the bulk cytosol. The basis for these differences between cytosolic and membrane-bound forms of PDE4 is not understood, but could include an effect of interacting proteins on UCR2 gating or on PDE4 dimer formation.

Recently, we have shown that PDE4D selective allosteric modulators have similar efficacy and slightly greater potency than rolipram in tests of short- and long-term memory, Table 4 (Burgin et al. 2010). Before the interest in the use of PDE4 inhibitors for treating anti-inflammatory conditions, the prototypical PDE4 selective inhibitor, rolipram, was explored in animal models and human clinical trials for the treatment of depression (Wachtel 1983; Zeller et al. 1984; Bobon et al. 1988; Laux et al. 1988; Hebenstreit et al. 1989; Scott et al. 1991; Fleischhacker et al. 1992). However, the nausea/emesis side-effect profile led to a cessation of interest in this. Interestingly, there recently has been a resurgence of interest in this use for PDE4 inhibitors, which have again now entered clinical development to treat depression and improve cognition (Tully et al. 2003). With regard to the antidepressant effect in animal models, PDE4 inhibitors have immediate effects on behavior (Zhang et al. 2006) as well as long-term effects on growth factor expression in brain (Fujimaki et al. 2000) and hippocampal neurogenesis (Li et al. 2009). Indeed, rolipram has benefit in multiple animal models of short- and long-term memory formation (Blokland et al. 2006), enhances long-term potentiation of synaptic efficacy (Vecsey et al. 2009), and is protective in transgenic models of Alzheimer's disease (Gong et al. 2004; Smith et al. 2009) and Huntington's disease (DeMarch 2008). PDE4 modulates cAMP-dependent phosphorylation of the CREB transcription factor, a pathway critical for long-term memory formation

Table 4 Procognitive benefit of rolipram and a PDE4D selective, allosteric modulator in comparison to emetic dose in *Suncus murinus* (Asian house shrew), the beagle dog, and cynomolgus monkey

Procognitive benefit in mice		
Test	Rolipram MED (mg/kg)	D159687 MED (mg/kg)
Scopolamine impaired Y-maze	0.03	0.01
Novel object recognition after 24 h	0.10	0.03
Emetic threshold		
Species	Rolipram NOEL (mg/kg)	D159687 NOEL (mg/kg)
<i>Suncus murinus</i>	0.10	10
Beagle dog	0.10	300
Cynomolgus monkey	0.02	10

Compounds were dosed orally. The minimum effective dose (MED) for procognitive benefit was the lowest dose at which discrimination in the Y-maze or NOR test was statistically significant (Burgin et al. 2010). The No Observable Effect Level (NOEL) for emesis was the maximum dose at which no effect on emesis was observed. Rolipram data in *Suncus* are from (Hirose et al. 2007), rolipram data in dog are from (Heaslip and Evans 1995), and the rolipram emetic threshold in the cynomolgus monkey is projected based on allometric scaling from human (Hebenstreit et al. 1989)

(Bailey et al. 1996). CREB regulates a transcriptional cascade ultimately leading to the remodeling of synaptic structure (Tully et al. 2003).

6 Improved Tolerability of UCR2-Directed, PDE4 Allosteric Modulators

The emetic response to PDE4 inhibitors is mediated in part by a brainstem noradrenergic pathway (Robichaud et al. 2001) and tolerability can be improved by limiting distribution to brain (Aoki et al. 2001). Until recently, subtype selective PDE4 inhibitors were not available, so it was not possible to link emesis with inhibition of a particular PDE4 subtype. Although PDE4 gene-deleted mice have been created by Conti and coworkers (Jin et al. 1999; Jin and Conti 2002), emesis cannot be explored directly in mice. Unlike humans, rodents cannot relax their crural sling, a band of muscles closing off the esophagus, while forcefully contracting their diaphragm, so they are unable to vomit. Since vomiting cannot be assessed directly, reduction of the duration of ketamine/xylazine anesthesia has been introduced as a behavioral correlate of emesis that is sensitive to PDE4 inhibition (Robichaud et al. 2002a). PDE4D null mice, but not PDE4B null mice show a shortening of anesthesia duration in this test, suggesting that inhibition of PDE4D may be the cause of emesis in nonrodent species (Robichaud et al. 2002b). This is in contrast to the emetic effect reported for highly selective PDE4D and PDE4B inhibitors. Chambers et al. reported nicotinamide-based inhibitors that are at least 25× selective for PDE4D (Chambers et al. 2006), which later were found to be emetic in animals and humans (unpublished). Naganuma et al. reported 2-arylpyrimidine-based inhibitors that are up to 100× selective for PDE4B; however, the lead compound was emetic in ferrets (Naganuma et al. 2009). Transcripts for both PDE4D and PDE4B can be detected in brainstem emetic centers of rodents and human (Perez-Torres et al. 2000), so the simple conclusion that inhibition of PDE4D is the cause of emesis based on the Robichaud data in mice (Robichaud et al. 2002b) may not be correct.

We found it possible to design potent UCR2-directed compounds with high selectivity for PDE4D (>100-fold). Comparing compounds with full and partial inhibition of PDE4D, full inhibitors reduced anesthesia in the ketamine/xylazine anesthesia test while partial inhibitors did not, even when dosed intravenously at a 1,000-fold greater dose than needed for procognitive benefit in the least sensitive cognitive test (Burgin et al. 2010). Emetic potential was investigated further in *Suncus murinus* (Asian house shrew), the beagle dog and cynomolgus monkey. *Suncus* have an emetic response to motion, ethanol overdose, and many classes of drugs that are emetic in human (Ueno et al. 1987; Hirose et al. 2007). A UCR2-directed, full inhibitor with >100-fold selectivity for PDE4D over PDE4B (D157140) caused emesis at 0.1 mg/kg, indicating that potent PDE4D inhibitors are emetic (Robichaud et al. 2002b). In contrast, the clinical lead compound,

D159687, a UCR2-directed, partial inhibitor was 300-fold less emetic than the D157140 or rolipram, despite equal potency in cognition assays (Table 4). D159687 was 3,000-fold less emetic than rolipram in the beagle dog, and 500-fold less emetic in monkey. Thus, there is a striking difference between full and partial PDE4D selective-inhibitors directed against UCR2 with respect to tolerability. As discussed above, PDE4 allosteric modulators have less impact on cAMP hydrolysis in a cellular model than traditional, active site-directed compounds. Allosteric modulators that only partially inhibit PDE4D activity may achieve improved tolerability by maintaining temporal and spatial aspects of cAMP signaling.

7 C-Terminal-Directed Inhibitors Bind a Closed, Symmetric PDE4 Conformer

It also may be possible to exploit for drug design a symmetric conformer of PDE4 in which the C-terminal helices cap both active sites (Fig. 3). Naganuma and colleagues recently reported the discovery of PDE4 inhibitors that are >100-fold selective for PDE4B over PDE4D (Naganuma et al. 2009). The chemical series explored by Naganuma utilized a pyrimidine core, which provides a ring nitrogen able to hydrogen bond to the invariant, active site glutamine, as well as a planar aromatic scaffold that will be sandwiched by the active site P-clamp. Their pyrimidine series additionally explored the SAR around a pair of aromatic arms, which our molecular superposition studies suggest are similar to the chemotype described above for UCR2-directed, allosteric modulators (Fig. 5).

Our studies of Naganuma compound 33 indicate that it likely interacts with the C-terminus of PDE4B, thereby achieving selectivity and potency (Fig. 5). We compared activity of compound 33 against PDE4B5, a supershort isoform, and a truncation lacking the C-terminus. This demonstrated that the compound required the PDE4B C-terminus and not the PDE4B UCR2 for potency and subtype selectivity. We think it likely that compound 33 exploits a binding pose similar to that which we reported for PMNPQ (PDB ID: 3G45) and a C-terminal PDE4D regulatory helix (Burgin et al. 2010). In that structure, the aromatic arms of PMPQ clamps a phenylalanine in a C-terminal helix that caps the active site. The C-terminal helix spans residues Gly593 to Thr603 (GQTEKFQFELTL) and contains a conserved FQF motif. The region is homologous to Gly673 to Thr683 in PDE4B3. Thus, we think it likely that compound 33 is acting as a bifunctional intramolecular clamp bridging Gln615 in the PDE4B active site and an aromatic residue, likely a phenylalanine on an α -helix in the PDE4B C-terminus.

Naganuma et al. report that compound 33 inhibits TNF α production in mice *in vitro* and *in vivo* (Naganuma et al. 2009). In mice, it decreases LPS-induced TNF α production with a 50% inhibitory oral dose of 14 mg/kg. The compound also decreases LPS-induced neutrophil migration to lung in ferrets at a 50% inhibitory dose of 12.5–25 mg/kg. In ferrets, the NOEL for emesis is reported as an oral dose

of 100 mg/kg. Thus, the therapeutic margin in ferret is four- to tenfold. This is the first exploration of PDE4B pharmacology in preclinical models. Although compound 33 did cause emesis in ferret at a high dose, the compound is only ~120× selective for PDE4B over PDE4D. Thus, it is not clear whether PDE4B inhibition was producing emesis or whether the PDE4D inhibition was sufficient to reach a threshold for emesis. Perhaps, compounds with even greater selectivity for PDE4B will be found to have proportionately lower potential for emesis.

8 Conclusions

Our new structures of the PDE4 catalytic unit include, for the first time, the key portion of UCR2, which characterizes PDE4 super-short isoforms. This provides a functional regulatory domain, where we demonstrate (Burgin et al 2010) that this portion of UCR2 is able to cap the PDE4 active site. In this, we develop a dual-gating model of PDE4 regulation where enzyme activity is controlled by such “capping” in a way that can be regulated by previously described changes in the phosphorylation status of the enzyme and fine-tuned by interaction with specific accessory proteins.

Three different PDE4 conformers have been identified that can be exploited for the design of subtype-selective or kinetically novel PDE4 allosteric modulators. The first is an open conformer in which both active sites are accessible to substrate and are catalytically independent. The second is a symmetrical, closed conformer in which a C-terminal regulatory helix closes intramolecularly across the active of each PDE4 monomer. In this conformation, the enzyme is completely inhibited with both active sites closed to cAMP. The third is an asymmetric PDE4 dimer in which one UCR2 is closed intermolecularly across the active site of the opposite monomer.

PDE4 subtype selectivity can be achieved by designing compounds that interact selectively with the gating helices in UCR2 or at the C-terminus. Subtype-selectivity can be achieved in the case of the UCR2-binding site by exploiting the Phe/Tyr polymorphism in PDE4D that is unique to primates. How subtype-selectivity is achieved by compounds that engage the C-terminal gating helix (Naganuma et al. 2009) awaits detailed structural information.

In the open PDE4 conformer, the traditional approach to the design of competitive, active site-directed inhibitors has yielded potent compounds that nonselectively inhibit all PDE4 subtypes. PDE4 competitive inhibitors have shown therapeutic benefit in human clinical trials, primarily in respiratory diseases such as asthma and chronic obstructive pulmonary disorder (Giembycz 2002; Lipworth 2005). However, the clinical dose for such actions of these compounds appears to be limited by emesis.

By weakening the interaction with the active site P-clamp, UCR2-directed, allosteric modulators can be designed that only partially inhibit PDE4 hydrolysis of cAMP. As only one active site can be capped in the dimer, allosteric modulators

exploiting this binding pose cause partial inhibition of activity. UCR2 capping of one active site not only blocks catalysis at that site but also decreases the turnover rate at the other active site. This explains why the maximum inhibition is $>50\%$. Such compounds are potent in cellular and *in vivo* assays, but have less effect on cellular cAMP levels than do competitive PDE4 inhibitors. The intrinsic ceiling on the magnitude of PDE4 inhibition may better maintain spatial and temporal patterning of signaling in cAMP microdomains with the consequence that tolerability is improved.

We showed that UCR2 capping affects cAMP hydrolysis as the Phe196/Tyr274 polymorphism affects apparent K_M (Burgin et al. 2010). Given such insights, one wonders whether endogenous ligands, other than cAMP, that modulate PDE4 activity in a cellular context exist. The gating mechanism for regulating PDE4 activity is probably general to other families of PDE as many of these contain unique upstream regulatory domains (e.g., GAF domains in PDE2, 5, 6, 10, and 11; Ca^{2+} /calmodulin domains in PDE1; the PAS domain in PDE8) (Lugnier 2006).

Pandit et al. have reported structures of the PDE2 catalytic domain with the GAF regulatory domain (Pandit et al. 2009). cGMP binding to the GAF domains induces an allosteric shift in the PDE2 dimer interface that allows a regulatory helix to uncap the active site, thereby allowing access of substrate. Sequence alignment of PDE7 with PDE4 reveals homology across the UCR2 helix, inspection of the PDE7 catalytic domain (PDB ID: 3G3N) reveals a groove across the active site as in PDE4 suggestive of a capping site for a regulatory helix, and indeed, the PDE4 UCR2 regulatory helix can be docked into that groove *in silico* (Burgin, unpublished). Thus, in PDE4, PDE2 and probably in PDE7, regulatory helices may control enzymatic activity by gating access of substrate to the active site. PDE activity may be fine tuned further by accessory proteins binding closed or open PDE conformers.

References

- Aoki M, Fukunaga M, Sugimoto T, Hirano Y, Kobayashi M, Honda K, Yamada T (2001) Studies on mechanisms of low emetogenicity of YM976, a novel phosphodiesterase type 4 inhibitor. *J Pharmacol Exp Ther* 298:1142–1149
- Asirvatham AL, Galligan SG, Schillace RV, Davey MP, Vasta V, Beavo JA, Carr DW (2004) A-kinase anchoring proteins interact with phosphodiesterases in T lymphocyte cell lines. *J Immunol* 173:4806–4814
- Bailey CH, Bartsch D, Kandel ER (1996) Toward a molecular definition of long-term memory storage. *Proc Natl Acad Sci U S A* 93:13445–13452
- Baillie GS, Adams DR, Bhari N, Houslay TM, Vadrevu S, Meng D, Li X, Dunlop A, Milligan G, Bolger GB, Klussmann E, Houslay MD (2007) Mapping binding sites for the PDE4D5 cAMP-specific phosphodiesterase to the N- and C-domains of beta-arrestin using spot-immobilized peptide arrays. *Biochem J* 404:71–80
- Beard MB, Olsen AE, Jones RE, Erdogan S, Houslay MD, Bolger GB (2000) UCR1 and UCR2 domains unique to the cAMP-specific phosphodiesterase family form a discrete module via electrostatic interactions. *J Biol Chem* 275:10349–10358

- Blackwood DH, Muir WJ (2004) Clinical phenotypes associated with DISC1, a candidate gene for schizophrenia. *Neurotox Res* 6:35–41
- Blokland A, Schreiber R, Prickaerts J (2006) Improving memory: a role for phosphodiesterases. *Curr Pharm Des* 12:2511–2523
- Bobon D, Breulet M, Gerard-Vandenhove MA, Guiot-Goffioul F, Plomteux G, Sastre-Hernandez M, Schratzter M, Troisfontaines B, von Frenckell R, Wachtel H (1988) Is phosphodiesterase inhibition a new mechanism of antidepressant action? A double blind double-dummy study between rolipram and desipramine in hospitalized major and/or endogenous depressives. *Eur Arch Psychiatry Neurol Sci* 238:2–6
- Bolger G, Michaeli T, Martins T, St John T, Steiner B, Rodgers L, Riggs M, Wigler M, Ferguson K (1993) A family of human phosphodiesterases homologous to the dunce learning and memory gene product of *Drosophila melanogaster* are potential targets for antidepressant drugs. *Mol Cell Biol* 13:6558–6571
- Bolger GB, McCahill A, Huston E, Cheung YF, McSorley T, Baillie GS, Houslay MD (2003a) The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with beta-arrestins. *J Biol Chem* 278:49230–49238
- Bolger GB, Peden AH, Steele MR, MacKenzie C, McEwan DG, Wallace DA, Huston E, Baillie GS, Houslay MD (2003b) Attenuation of the activity of the cAMP-specific phosphodiesterase PDE4A5 by interaction with the immunophilin XAP2. *J Biol Chem* 278:33351–33363
- Bolger GB, Baillie GS, Li X, Lynch MJ, Herzyk P, Mohamed A, Mitchell LH, McCahill A, Hundsrucker C, Klussmann E, Adams DR, Houslay MD (2006) Scanning peptide array analyses identify overlapping binding sites for the signalling scaffold proteins, beta-arrestin and RACK1, in cAMP-specific phosphodiesterase PDE4D5. *Biochem J* 398:23–36
- Brideau C, Van Staden C, Styhler A, Rodger IW, Chan CC (1999) The effects of phosphodiesterase type 4 inhibitors on tumour necrosis factor-alpha and leukotriene B4 in a novel human whole blood assay. *Br J Pharmacol* 126:979–988
- Burgin AB, Magnusson OT, Singh J, Bjornsson JM, Thorsteinsdottir M, Hrafnisdottir S, Hagen T, Witte P, Staker BL, Kiselyov AS, Stewart LJ, Gurney ME (2010) Design of Phosphodiesterase Type 4D (PDE4D) allosteric modulators for cognition with improved safety. *Nat Biotechnol* 28:63–70
- Card GL, England BP, Suzuki Y, Fong D, Powell B, Lee B, Luu C, Tabrizid M, Gillette S, Ibrahim PN, Artis DR, Bollag G, Milburn MV, Kim SH, Schlessinger J, Zhang KY (2004) Structural basis for the activity of drugs that inhibit phosphodiesterases. *Structure* 12:2233–2247
- Carlisle Michel JJ, Dodge KL, Wong W, Mayer NC, Langeberg LK, Scott JD (2004) PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. *Biochem J* 381:587–592
- Castro LR, Gervasi N, Guiot E, Cavellini L, Nikolaev VO, Paupardin-Tritsch D, Vincent P (2010) Type 4 phosphodiesterase plays different integrating roles in different cellular domains in pyramidal cortical neurons. *J Neurosci* 30:6143–6151
- Chambers RJ, Abrams K, Castleberry TA, Cheng JB, Fisher DA, Kamath AV, Marfat A, Nettleton DO, Pillar JD, Salter ED, Sheils AL, Shirley JT, Turner CR, Umland JP, Lam KT (2006) A new chemical tool for exploring the role of the PDE4D isozyme in leukocyte function. *Bioorg Med Chem Lett* 16:718–721
- Charlie NK, Thomure AM, Schade MA, Miller KG (2006) The Dunce cAMP phosphodiesterase PDE-4 negatively regulates G alpha(s)-dependent and G alpha(s)-independent cAMP pools in the *Caenorhabditis elegans* synaptic signaling network. *Genetics* 173:111–130
- Chen CN, Denome S, Davis RL (1986) Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the *Drosophila dunce +* gene, the structural gene for cAMP phosphodiesterase. *Proc Natl Acad Sci U S A* 83:9313–9317
- Clapcote SJ, Lipina TV, Millar JK, Mackie S, Christie S, Ogawa F, Lerch JP, Trimble K, Uchiyama M, Sakuraba Y, Kaneda H, Shiroishi T, Houslay MD, Henkelman RM, Sled JG,

- Gondo Y, Porteous DJ, Roder JC (2007) Behavioral phenotypes of *Disc1* missense mutations in mice. *Neuron* 54:387–402
- Conti M, Iona S, Cuomo M, Swinnen JV, Odeh J, Svoboda ME (1995) Characterization of a hormone-inducible, high affinity adenosine 3'-5'-cyclic monophosphate phosphodiesterase from the rat Sertoli cell. *Biochemistry* 34:7979–7987
- Cooper DM (2003) Regulation and organization of adenylyl cyclases and cAMP. *Biochem J* 375:517–529
- Davis RL, Takayasu H, Eberwine M, Myres J (1989) Cloning and characterization of mammalian homologs of the *Drosophila dunce +* gene. *Proc Natl Acad Sci U S A* 86:3604–3608
- Day JP, Dow JA, Houslay MD, Davies SA (2005) Cyclic nucleotide phosphodiesterases in *Drosophila melanogaster*. *Biochem J* 388:333–342
- DeMarch Z, Giampà C, Patassini S, Bernardi G, Fusco FR (2008) Beneficial effects of rolipram in the R6/2 mouse model of Huntington's disease. *Neurobiol Dis.* Jun;30(3):375–387 Epub 2008 Mar 7. PMID:18424161
- Fleischhacker WW, Hinterhuber H, Bauer H, Pflug B, Berner P, Simhandl C, Wolf R, Gerlach W, Jaklitsch H, Sastre-y-Hernandez M et al (1992) A multicenter double-blind study of three different doses of the new cAMP-phosphodiesterase inhibitor rolipram in patients with major depressive disorder. *Neuropsychobiology* 26:59–64
- Fujimaki K, Morinobu S, Duman RS (2000) Administration of a cAMP phosphodiesterase 4 inhibitor enhances antidepressant-induction of BDNF mRNA in rat hippocampus. *Neuropharmacology* 22:42–51
- Giembycz MA (2002) Development status of second generation PDE4 inhibitors for asthma and COPD: the story so far. *Monaldi Arch Chest Dis* 57:48–64
- Giembycz MA (2005) Phosphodiesterase-4: selective and dual-specificity inhibitors for the therapy of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2:326–333, discussion 340–321
- Giembycz MA (2006) An update and appraisal of the cilomilast Phase III clinical development programme for chronic obstructive pulmonary disease. *Br J Clin Pharmacol* 62:138–152
- Gong B, Vitolo OV, Trinchese F, Liu S, Shelanski M, Arancio O (2004) Persistent improvement in synaptic and cognitive functions in an Alzheimer mouse model after rolipram treatment. *J Clin Invest* 114:1624–1634
- Hatzelmann A, Schudt C (2001) Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *J Pharmacol Exp Ther* 297:267–279
- Heaslip RJ, Evans DY (1995) Emetic, central nervous system, and pulmonary activities of rolipram in the dog. *Eur J Pharmacol* 286:281–290
- Hebenstreit GF, Fellerer K, Fichte K, Fischer G, Geyer N, Meya U, Sastre-y-Hernandez M, Schony W, Schratzer M, Soukop W et al (1989) Rolipram in major depressive disorder: results of a double-blind comparative study with imipramine. *Pharmacopsychiatry* 22:156–160
- Henkel-Tigges J, Davis RL (1990) Rat homologs of the *Drosophila dunce* gene code for cyclic AMP phosphodiesterases sensitive to rolipram and RO 20-1724. *Mol Pharmacol* 37:7–10
- Hirose R, Manabe H, Nonaka H, Yanagawa K, Akuta K, Sato S, Ohshima E, Ichimura M (2007) Correlation between emetic effect of phosphodiesterase 4 inhibitors and their occupation of the high-affinity rolipram binding site in *Suncus murinus* brain. *Eur J Pharmacol* 573:93–99
- Hoffmann R, Wilkinson IR, McCallum JF, Engels P, Houslay MD (1998) cAMP-specific phosphodiesterase HSPDE4D3 mutants which mimic activation and changes in rolipram inhibition triggered by protein kinase A phosphorylation of Ser-54: generation of a molecular model. *Biochem J* 333(Pt 1):139–149
- Hoffmann R, Baillie GS, MacKenzie SJ, Yarwood SJ, Houslay MD (1999) The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. *EMBO J* 18:893–903
- Houslay MD (2001) PDE4 cAMP-specific phosphodiesterases. *Prog Nucleic Acid Res Mol Biol* 69:249–315

- Houslay MD, Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J* 370: 1–18
- Houslay MD, Adams DR (2010) Putting the lid on phosphodiesterase 4. *Nat Biotechnol* 28:38–40
- Houslay MD, Schafer P, Zhang KY (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10:1503–1519
- Houslay MD, Baillie GS, Maurice DH (2007) cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. *Circ Res* 100:950–966
- Huston E, Pooley L, Julien P, Scotland G, McPhee I, Sullivan M, Bolger G, Houslay MD (1996) The human cyclic AMP-specific phosphodiesterase PDE-46 (HSPDE4A4B) expressed in transfected COS7 cells occurs as both particulate and cytosolic species that exhibit distinct kinetics of inhibition by the antidepressant rolipram. *J Biol Chem* 271:31334–31344
- Jacobitz S, McLaughlin MM, Livi GP, Burman M, Torphy TJ (1996) Mapping the functional domains of human recombinant phosphodiesterase 4A: structural requirements for catalytic activity and rolipram binding. *Mol Pharmacol* 50:891–899
- Jin SL, Conti M (2002) Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-alpha responses. *Proc Natl Acad Sci U S A* 99:7628–7633
- Jin SL, Richard FJ, Kuo WP, D'Ercole AJ, Conti M (1999) Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice. *Proc Natl Acad Sci U S A* 96:11998–12003
- Ke H, Wang H (2007) Crystal structures of phosphodiesterases and implications on substrate specificity and inhibitor selectivity. *Curr Top Med Chem* 7:391–403
- Kovala T, Sanwal BD, Ball EH (1997) Recombinant expression of a type IV, cAMP-specific phosphodiesterase: characterization and structure-function studies of deletion mutants. *Biochemistry* 36:2968–2976
- Kranz M, Wall M, Evans B, Miah A, Ballantine S, Delves C, Dombroski B, Gross J, Schneck J, Villa JP, Neu M, Somers DO (2009) Identification of PDE4B Over 4D subtype-selective inhibitors revealing an unprecedented binding mode. *Bioorg Med Chem* 17:5336–5341
- Laux G, Becker T, Kuhne G, Lesch KP, Riederer P, Beckmann H (1988) Clinical and biochemical effects of the selective phosphodiesterase inhibitor rolipram in depressed inpatients controlled by determination of plasma level. *Pharmacopsychiatry* 21:378–379
- Lee ME, Markowitz J, Lee JO, Lee H (2002) Crystal structure of phosphodiesterase 4D and inhibitor complex(1). *FEBS Lett* 530:53–58
- Lehnart SE, Wehrens XH, Reiken S, Warriar S, Belevych AE, Harvey RD, Richter W, Jin SL, Conti M, Marks AR (2005) Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. *Cell* 123:25–35
- Li YF, Huang Y, Amsdell SL, Xiao L, O'Donnell JM, Zhang HT (2009) Antidepressant- and anxiolytic-like effects of the phosphodiesterase-4 inhibitor rolipram on behavior depend on cyclic AMP response element binding protein-mediated neurogenesis in the hippocampus. *Neuropsychopharmacology* 34:2404–2419
- Lipworth BJ (2005) Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease. *Lancet* 365:167–175
- Liu S, Laliberte F, Bobechko B, Bartlett A, Lario P, Gorseth E, Van Hamme J, Gresser MJ, Huang Z (2001) Dissecting the cofactor-dependent and independent bindings of PDE4 inhibitors. *Biochemistry* 40:10179–10186
- Lorimer D, Raymond A, Walchli J, Mixon M, Wallace E, Barrow A, Grice R, Burgin A, Stewart L (2009) Gene composer: database software for protein construct design, codon engineering, and gene synthesis. *BMC Biotechnol* 9:36
- Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 109:366–398
- Lynch MJ, Baillie GS, Mohamed A, Li X, Maisonneuve C, Klussmann E, van Heeke G, Houslay MD (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated

- switching of the beta2-adrenergic receptor to activation of ERK in HEK293B2 cells. *J Biol Chem* 280:33178–33189
- MacKenzie SJ, Baillie GS, McPhee I, Bolger GB, Houslay MD (2000) ERK2 mitogen-activated protein kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterases. The involvement of COOH-terminal docking sites and NH2-terminal UCR regions. *J Biol Chem* 275:16609–16617
- McPhee I, Yarwood SJ, Scotland G, Huston E, Beard MB, Ross AH, Houslay ES, Houslay MD (1999) Association with the SRC family tyrosyl kinase LYN triggers a conformational change in the catalytic region of human cAMP-specific phosphodiesterase HSPDE4A4B. Consequences for rolipram inhibition. *J Biol Chem* 274:11796–11810
- Millar JK, Pickard BS, Mackie S, James R, Christie S, Buchanan SR, Malloy MP, Chubb JE, Huston E, Baillie GS, Thomson PA, Hill EV, Brandon NJ, Rain JC, Camargo LM, Whiting PJ, Houslay MD, Blackwood DH, Muir WJ, Porteous DJ (2005) DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. *Science* 310:1187–1191
- Monaco L, Vicini E, Conti M (1994) Structure of two rat genes coding for closely related rolipram-sensitive cAMP phosphodiesterases. Multiple mRNA variants originate from alternative splicing and multiple start sites. *J Biol Chem* 269:347–357
- Murdoch H, Mackie S, Collins DM, Hill EV, Bolger GB, Klussmann E, Porteous DJ, Millar JK, Houslay MD (2007) Isoform-selective susceptibility of DISC1/phosphodiesterase-4 complexes to dissociation by elevated intracellular cAMP levels. *J Neurosci* 27:9513–9524
- Naganuma K, Omura A, Maekawara N, Saitoh M, Ohkawa N, Kubota T, Nagumo H, Kodama T, Takemura M, Ohtsuka Y, Nakamura J, Tsujita R, Kawasaki K, Yokoi H, Kawanishi M (2009) Discovery of selective PDE4B inhibitors. *Bioorg Med Chem Lett* 19:3174–3176
- Pandit J, Forman MD, Fennell KF, Dillman KS, Menniti FS (2009) Mechanism for the allosteric regulation of phosphodiesterase 2A deduced from the X-ray structure of a near full-length construct. *Proc Natl Acad Sci U S A* 106:18225–18230
- Perez-Torres S, Miro X, Palacios JM, Cortes R, Puigdomenech P, Mengod G (2000) Phosphodiesterase type 4 isozymes expression in human brain examined by in situ hybridization histochemistry and [³H]rolipram binding autoradiography. Comparison with monkey and rat brain. *J Chem Neuroanat* 20:349–374
- Raymond A, Lovell S, Lorimer D, Walchli J, Mixon M, Wallace E, Thompkins K, Archer K, Burgin A, Stewart L (2009) Combined protein construct and synthetic gene engineering for heterologous protein expression and crystallization using Gene Composer. *BMC Biotechnol* 9:37
- Reid P (2002) Roflumilast Altana Pharma. *Curr Opin Investig Drugs* 3:1165–1170
- Richter W, Conti M (2002) Dimerization of the type 4 cAMP-specific phosphodiesterases is mediated by the upstream conserved regions (UCRs). *J Biol Chem* 277:40212–40221
- Robichaud A, Tattersall FD, Choudhury I, Rodger IW (1999) Emesis induced by inhibitors of type IV cyclic nucleotide phosphodiesterase (PDE IV) in the ferret. *Neuropharmacology* 38:289–297
- Robichaud A, Savoie C, Stamatou PB, Tattersall FD, Chan CC (2001) PDE4 inhibitors induce emesis in ferrets via a noradrenergic pathway. *Neuropharmacology* 40:262–269
- Robichaud A, Savoie C, Stamatou PB, Lachance N, Jolicoeur P, Rasori R, Chan CC (2002a) Assessing the emetic potential of PDE4 inhibitors in rats. *Br J Pharmacol* 135:113–118
- Robichaud A, Stamatou PB, Jin SL, Lachance N, MacDonald D, Laliberte F, Liu S, Huang Z, Conti M, Chan CC (2002b) Deletion of phosphodiesterase 4D in mice shortens alpha(2)-adrenoceptor-mediated anesthesia, a behavioral correlate of emesis. *J Clin Invest* 110:1045–1052
- Rocque WJ, Tian G, Wiseman JS, Holmes WD, Zajac-Thompson I, Willard DH, Patel IR, Wisely GB, Clay WC, Kadwell SH, Hoffman CR, Luther MA (1997) Human recombinant phosphodiesterase 4B2B binds (R)-rolipram at a single site with two affinities. *Biochemistry* 36:14250–14261

- Saldou N, Obermolte R, Huber A, Baecker PA, Wilhelm R, Alvarez R, Li B, Xia L, Callan O, Su C, Jarnagin K, Shelton ER (1998) Comparison of recombinant human PDE4 isoforms: interaction with substrate and inhibitors. *Cell Signal* 10:427–440
- Scott AI, Perini AF, Shering PA, Whalley LJ (1991) In-patient major depression: is rolipram as effective as amitriptyline? *Eur J Clin Pharmacol* 40:127–129
- Sette C, Conti M (1996) Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation. *J Biol Chem* 271:16526–16534
- Smith DL, Pozueta J, Gong B, Arancio O, Shelanski M (2009) Reversal of long-term dendritic spine alterations in Alzheimer disease models. *Proc Natl Acad Sci U S A* 106:16877–16882
- Souness JE, Rao S (1997) Proposal for pharmacologically distinct conformers of PDE4 cyclic AMP phosphodiesterases. *Cell Signal* 9:227–236
- Spina D (2008) PDE4 inhibitors: current status. *Br J Pharmacol* 155:308–315
- Stefan E, Wiesner B, Baillie GS, Mollajew R, Henn V, Lorenz D, Furkert J, Santamaria K, Nedvetsky P, Hundsrucker C, Beyermann M, Krause E, Pohl P, Gall I, MacIntyre AN, Bachmann S, Houslay MD, Rosenthal W, Klussmann E (2007) Compartmentalization of cAMP-dependent signaling by phosphodiesterase-4D is involved in the regulation of vasopressin-mediated water reabsorption in renal principal cells. *J Am Soc Nephrol* 18:199–212
- Swinnen JV, Joseph DR, Conti M (1989) Molecular cloning of rat homologues of the *Drosophila melanogaster* *dunce* cAMP phosphodiesterase: evidence for a family of genes. *Proc Natl Acad Sci U S A* 86:5325–5329
- Terrenoire C, Houslay MD, Baillie GS, Kass RS (2009) The cardiac IKs potassium channel macromolecular complex includes the phosphodiesterase PDE4D3. *J Biol Chem* 284:9140–9146
- Terrin A, Di Benedetto G, Pertegato V, Cheung YF, Baillie G, Lynch MJ, Elvassore N, Prinz A, Herberg FW, Houslay MD, Zaccolo M (2006) PGE(1) stimulation of HEK293 cells generates multiple contiguous domains with different [cAMP]: role of compartmentalized phosphodiesterases. *J Cell Biol* 175:441–451
- Tully T, Bourchouladze R, Scott R, Tallman J (2003) Targeting the CREB pathway for memory enhancers. *Nat Rev Drug Discov* 2:267–277
- Ueno S, Matsuki N, Saito H (1987) *Suncus murinus*: a new experimental model in emesis research. *Life Sci* 41:513–518
- Vecsey CG, Baillie GS, Jaganath D, Havekes R, Daniels A, Wimmer M, Huang T, Brown KM, Li XY, Descalzi G, Kim SS, Chen T, Shang YZ, Zhuo M, Houslay MD, Abel T (2009) Sleep deprivation impairs cAMP signalling in the hippocampus. *Nature* 461:1122–1125
- Verde I, Pahlke G, Salanova M, Zhang G, Wang S, Coletti D, Onuffer J, Jin SL, Conti M (2001) Myomegalin is a novel protein of the golgi/centrosome that interacts with a cyclic nucleotide phosphodiesterase. *J Biol Chem* 276:11189–11198
- Wachtel H (1983) Potential antidepressant activity of rolipram and other selective cyclic adenosine 3', 5'-monophosphate phosphodiesterase inhibitors. *Neuropharmacology* 22:267–272
- Wang H, Peng MS, Chen Y, Geng J, Robinson H, Houslay MD, Cai J, Ke H (2007a) Structures of the four subfamilies of phosphodiesterase-4 provide insight into the selectivity of their inhibitors. *Biochem J* 408:193–201
- Wang H, Robinson H, Ke H (2007b) The molecular basis for different recognition of substrates by phosphodiesterase families 4 and 10. *J Mol Biol* 371:302–307
- Warrier S, Ramamurthy G, Eckert RL, Nikolaev VO, Lohse MJ, Harvey RD (2007) cAMP microdomains and L-type Ca²⁺ channel regulation in guinea-pig ventricular myocytes. *J Physiol* 580:765–776
- Wilhelm RS, Axt S (1995) 8-phenylcyclopentenoquinoline and 8-phenylcyclohexeneoquinoline derivatives. Syntex, Palo Alto, CA
- Willoughby D, Wong W, Schaack J, Scott JD, Cooper DM (2006) An anchored PKA and PDE4 complex regulates subplasmalemmal cAMP dynamics. *EMBO J* 25:2051–2061

- Xu RX, Hassell AM, Vanderwall D, Lambert MH, Holmes WD, Luther MA, Rocque WJ, Milburn MV, Zhao Y, Ke H, Nolte RT (2000) Atomic structure of PDE4: insights into phosphodiesterase mechanism and specificity. *Science* 288:1822–1825
- Yarwood SJ, Steele MR, Scotland G, Houslay MD, Bolger GB (1999) The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. *J Biol Chem* 274:14909–14917
- Zeller E, Stief HJ, Pflug B, Sastre-y-Hernandez M (1984) Results of a phase II study of the antidepressant effect of rolipram. *Pharmacopsychiatry* 17:188–190
- Zhang KY, Card GL, Suzuki Y, Artis DR, Fong D, Gillette S, Hsieh D, Neiman J, West BL, Zhang C, Milburn MV, Kim SH, Schlessinger J, Bollag G (2004) A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases. *Mol Cell* 15:279–286
- Zhang HT, Zhao Y, Huang Y, Deng C, Hopper AT, De Vivo M, Rose GM, O'Donnell JM (2006) Antidepressant-like effects of PDE4 inhibitors mediated by the high-affinity rolipram binding state (HARBS) of the phosphodiesterase-4 enzyme (PDE4) in rats. *Psychopharmacology (Berl)* 186:209–217
- Zhao Y, Zhang HT, O'Donnell JM (2003) Inhibitor binding to type 4 phosphodiesterase (PDE4) assessed using [³H]piclamilast and [³H]rolipram. *J Pharmacol Exp Ther* 305:565–572

Regulation of Endothelial Barrier Function by Cyclic Nucleotides: The Role of Phosphodiesterases

James Surapisitchat and Joseph A. Beavo

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Abstract The endothelium plays an important role in maintaining normal vascular function. Endothelial barrier dysfunction leading to increased permeability and vascular leakage is associated with several pathological conditions such as edema and sepsis. Thus, the development of drugs that improve endothelial barrier function is an active area of research. In this chapter, the current knowledge concerning the signaling pathways regulating endothelial barrier function is discussed with a focus on cyclic nucleotide second messengers (cAMP and cGMP) and cyclic nucleotide phosphodiesterases (PDEs). Both cAMP and cGMP have been shown

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to have differential effects on endothelial permeability in part due to the various effector molecules, crosstalk, and compartmentalization of cyclic nucleotide signaling. PDEs, by controlling the amplitude, duration, and localization of cyclic nucleotides, have been shown to play a critical role in regulating endothelial barrier function. Thus, PDEs are attractive drug targets for the treatment of disease states involving endothelial barrier dysfunction.

Keywords cAMP · cGMP · Endothelial barrier · Endothelial permeability · Phosphodiesterase

1 Introduction

One of the major functions of the endothelium is to act as an active barrier between the circulating blood and the underlying vessel wall and tissues (Mehta and Malik 2006; Michel and Curry 1999; van Hinsbergh 1997; van Nieuw Amerongen et al. 2001; van Nieuw Amerongen and van Hinsbergh 2002). The endothelium can respond to a variety of signals to regulate the passage of proteins, fluid, and solutes through the endothelial barrier. While oxygen, water, and solutes can pass through this barrier via diffusion, channels, and slits in the cellular junctions, macromolecules can cross the endothelial barrier via three different mechanisms. These include (1) transcellular passage through the cell via vesicles, (2) passage via pores in the cell membrane usually formed by fused vesicles at the apical and basolateral membrane, or (3) passage between endothelial cells paracellularly (Mehta and Malik 2006; van Hinsbergh 1997). Dysregulation of endothelial barrier function is characteristic of many diseases and pathological conditions including atherosclerosis, asthma, tumor growth, edema, and sepsis (van Hinsbergh 1997; van Nieuw Amerongen and van Hinsbergh 2002). Thus, understanding the mechanisms that regulate endothelial permeability may allow for the development of drugs that can enhance or decrease barrier function. In this chapter, we briefly summarize what is currently known about the various mechanisms and signaling pathways that regulate endothelial permeability by focusing on the role of cyclic nucleotide phosphodiesterases as a potential drug target for the regulation of endothelial barrier function.

2 Transcellular and Paracellular Transport of Molecules Through the Endothelial Barrier

Transport of macromolecules such as proteins, carbohydrates, and lipids across the endothelial barrier occurs transcellularly or paracellularly. Most studies of transcellular transport of macromolecules have focused on the role of caveolae and vesiculo-vacuolar organelles (VVOs) as the transport vesicles. The proteins and mechanisms involved share many similarities to those that regulate neuronal vesicle

trafficking. Caveolae are hypothesized to play a role in both receptor-mediated and nonreceptor-mediated transcellular transport of macromolecules across the endothelial barrier (Cohen et al. 2004). Caveolae were first identified by electron microscopy as “flask-shaped” invaginations of the plasma membrane. It is these invaginations that are hypothesized to form the vesicles that mediate transcellular transport of macromolecules across the endothelium (Komarova and Malik 2010). For example, albumin transcytosis is initiated by binding of albumin to the albumin-binding protein gp60 found in caveolae, resulting in endocytosis and transport of the caveolae through the endothelial cell (Mehta and Malik 2006). VVOs, unlike caveolae, are uncoated interconnected vesicles and vacuoles present in endothelial cells. VVOs have been shown to be able to participate in transcellular transport of macromolecules across the endothelium, but their regulation is unknown. Roles for dynamin, SNARE complexes, actin, and microtubules in the transport of VVOs through endothelial cells have been suggested (Komarova and Malik 2010; Mehta and Malik 2006). Transcellular transport has been suggested to be regulated in part by Src kinases, certain PKC isoforms, PI3 kinases, Ca^{2+} , and the albumin-binding protein gp60 (Komarova and Malik 2010; Mehta and Malik 2006).

Paracellular transport of macromolecules across the endothelial barrier is achieved by opening of intercellular gaps between cells. Endothelial cells are connected with each other by a variety of junctional proteins that form (a) tight, (b) adherent, and (c) gap junctions. Tight junctions consist of proteins such as claudins, occludin, and junctional adhesion molecules. These transmembrane proteins have extracellular domains that bind tightly to each other via homotypic or heterotypic bonds and are linked intracellularly to the actin cytoskeleton. They are also linked to a variety of signaling molecules such as PKC- ζ and VASP. Tight and adherent junctions form zipper-like structures between endothelial cells, while gap junction proteins such as connexons form transmembrane channels between endothelial cells.

Adherent junctions are comprised primarily by vascular endothelial (VE)-cadherin whose extracellular domains participate in transoligomeric binding between endothelial cells forming the junction. The cytoplasmic domain of VE-cadherin, like those of the proteins found in tight junctions, is also proposed to be associated with the actin cytoskeleton as well as a variety of signaling molecules such as β -catenin, RhoGTPases, VASP, casein kinase II, Src kinases, and phosphatases such as SHP-1.

Both tight and adherent junctions link endothelial cells together forming a barrier. It is thought that changes in binding of tight and adherent junction proteins function to regulate the opening and closing of gaps between endothelial cells allowing paracellular transport of macromolecules.

Gap junctions consist of transmembrane hydrophilic proteins called connexons. A connexon from each neighboring endothelial cell pair forms an intercellular pore that can be regulated by serine/threonine and tyrosine phosphorylation. The pores formed between cells by gap junctions allow for the propagation of transmembrane potentials as well as the exchange of signals between endothelial cells via second messengers such as Ca^{2+} and IP_3 (Komarova and Malik 2010; Mehta and Malik 2006).

3 Signaling Pathways Shown to Regulate Endothelial Permeability

There are a large number of studies on endothelial permeability focused on trying to unravel the mechanisms by which permeability is both increased and decreased and how these mechanisms are regulated. The majority of the studies have addressed paracellular mechanisms of barrier function. Similar to smooth muscle, endothelial cells have a contractile system that requires actin, nonmuscle myosin, ATP, calcium, and calmodulin. Stimulation of endothelial contraction leads to the formation of small gaps between endothelial cells that, in time, lead to increased permeability. A variety of agents, including thrombin, VEGF, bradykinin, and histamine, have been found to increase endothelial permeability via increases in intracellular calcium. As with smooth muscle, increases in intracellular calcium will lead to the activation of myosin light chain kinase (MLCK). MLCK can then phosphorylate myosin light chains leading to actin-myosin-mediated endothelial cell contraction.

In addition to pathways that increase calcium, activation of other signaling pathways can also increase endothelial cell contraction. Thrombin has been shown to activate the small GTPase, RhoA. RhoA, via activation of Rho kinase, inhibits PP1M, the phosphatase that dephosphorylates myosin light chain. Thus, thrombin, in addition to increasing calcium and phosphorylation of myosin light chain via MLCK, inhibits dephosphorylation of myosin light chain via a RhoA-mediated mechanism. The cumulative effect is an increase in contraction. Other agents known to increase endothelial permeability such as TNF- α and H₂O₂ have also been shown to regulate RhoA and thus the contractile function of endothelial cells. All of these agents have also been shown to activate a variety of kinases that can lead to changes in the phosphorylation status of junctional proteins and barrier function and also to changes in gene expression.

While there are several pharmacological agonists that have been shown to decrease endothelial barrier function, there are only a few endogenous factors that have been shown to increase barrier function. These include sphingosine-1-phosphate (S1P), angiotensin-1, and cAMP (Jho et al. 2005; Komarova et al. 2007; Moore et al. 1998; Satchell et al. 2004). S1P is a phospholipid formed by the phosphorylation of sphingosine by sphingosine kinase. S1P is found in and released from platelets. The lack of sphingosine lysase, a key regulator of S1P degradation, leads to S1P storage in platelet granules (Yatomi et al. 1995). Endothelial cells express G-coupled receptors for S1P and the endothelial differentiation gene (EDG) receptors, Edg-1, Edg-3, and Edg-5 (Ozaki et al. 2003). These receptors are Gi coupled and lead to activation of the small GTPase Rac and adherent junction assembly (Takuwa 2002).

Finally, angiotensin-1 is a ligand for the endothelial-specific tyrosine kinase receptor, Tie-2 (Tsigkos et al. 2003). Angiotensin-1 has been shown to inhibit permeability induced by thrombin, bradykinin, histamine, and VEGF (Pizurki et al. 2003). In studies with VEGF, angiotensin-1 was shown to prevent Ca²⁺ influx and

contraction (Jho et al. 2005). However, the detailed mechanisms of angiotensin-1 action on barrier function remain to be elucidated.

4 The Role of cAMP in the Regulation of Endothelial Barrier Function

While the study of S1P and angiotensin-1 as agonists of endothelial barrier enhancement is just beginning, the role of cAMP and agonists that increase this second messenger, such as β -adrenergic agonists and serotonin, has been studied extensively (Mehta and Malik 2006; van Hinsbergh 1997). In general, cAMP has been shown to improve barrier function and decrease permeability under both basal and stimulated conditions *in vitro* and *in vivo*. Until recently, it had been accepted that cAMP improves endothelial barrier function only via activation of PKA (Mehta and Malik 2006). PKA has been shown to inhibit thrombin-induced RhoA activation (Qiao et al. 2003). RhoA is a member of the superfamily of Rho GTPases whose activity is determined by the binding of GTP. PKA has been shown to directly phosphorylate and inhibit RhoA (Lang et al. 1996). Another possible mechanism by which PKA inhibits RhoA is via the phosphorylation of GTP dissociation inhibitor (GDI) (Qiao et al. 2008). Since RhoA has been implicated in inhibiting MLC phosphatase, which can result in increased MLC phosphorylation and contraction, PKA activation would lead to increased phosphatase activity, decreased MLC phosphorylation, and relaxation. This should improve barrier function. PKA has also been shown to phosphorylate and inhibit MLCK (Verin et al. 1998). By preventing MLC phosphorylation by MLCK, PKA can reduce endothelial cell contraction. These decreases in contraction caused by PKA presumably lead to improved tight and adherent junction stabilization, decreased gap formation, and improved barrier function.

More recently, another cAMP effector molecule, Epac, was also shown to mediate at least part of the barrier-enhancing actions of cAMP (Fukuhara et al. 2005; Kooistra et al. 2005; Wittchen et al. 2005). Epac1 and Epac2 are guanine nucleotide exchange factors for the small G protein Rap (Gloerich and Bos 2010). The discovery of Epac as a target of cAMP signaling explained the various effects of cAMP that could not be attributed to PKA and cyclic nucleotide-gated ion channels. It was demonstrated that Epac can also inhibit thrombin-induced RhoA activation (Cullere et al. 2005). Similar to PKA, Epac-mediated inhibition of RhoA activity presumably leads to decreased MLC phosphorylation, endothelial relaxation, and increased barrier function. The relative contributions of PKA and Epac in mediating the barrier-enhancing effects of cAMP are just now beginning to be determined. Of the early studies describing the role of Epac in mediating the barrier-enhancing effects of cAMP, only one attempted to rule out the involvement of PKA via the use of H89, a PKA inhibitor (Fukuhara et al. 2005). However, H89 has also been shown to inhibit other kinases including Rho kinase (Leemhuis et al. 2002; Murray 2008).

Thus, H89 inhibition of Rho kinase, which is downstream of RhoA and PKA (Qiao et al. 2008), may lead to decreased permeability and may not rule out the possible role of PKA in decreasing permeability. Kooistra et al. demonstrated the role of Epac in regulating endothelial cell permeability via the use of RNAi (Kooistra et al. 2005). Kooistra et al. also used Epac-specific analogues in combination with RNAi knockdown of Epac to demonstrate the role of Epac in regulating endothelial cell permeability. RhoA seems to be regulated by both PKA and Epac. The use of PKI, a specific inhibitor of PKA, was shown to partially reverse the inhibitory effect of cAMP on RhoA (Qiao et al. 2003). On the other hand, it was shown that an Epac-specific cAMP analogue, 8-pCPT-2'-O-Me-cAMP, could mimic completely the inhibitory effects of forskolin- and rolipram (PDE4-specific inhibitor)-induced cAMP on RhoA, suggesting that the effects of cAMP are via Epac (Cullere et al. 2005). Further studies are needed to clarify the relative roles of PKA and Epac in mediating the inhibitory effects of cAMP on RhoA. Unpublished data from our laboratory suggest that the barrier-enhancing effects of cAMP may be due to both PKA and Epac. We found that inhibition of PKA by Rp-8-Br-cAMP increased thrombin-induced permeability, suggesting a role for PKA in preventing decreases in barrier function (Fig. 1). However, forskolin was still able to attenuate thrombin-induced permeability, and low doses of ANP, via inhibition of PDE3, were able to potentiate this effect, suggesting that another effector molecule also mediates the barrier-enhancing effect of cAMP (Surapisitchat et al. 2007). We also found that the activation of Epac was able to decrease thrombin-induced permeability using the Epac-specific activator, 8-CPT-2-O-ME-cAMP (Fig. 2). These results suggest that there may be different pools of cAMP mediating the effects of PKA and Epac on permeability. A recent report has demonstrated that cAMP can act via both PKA and

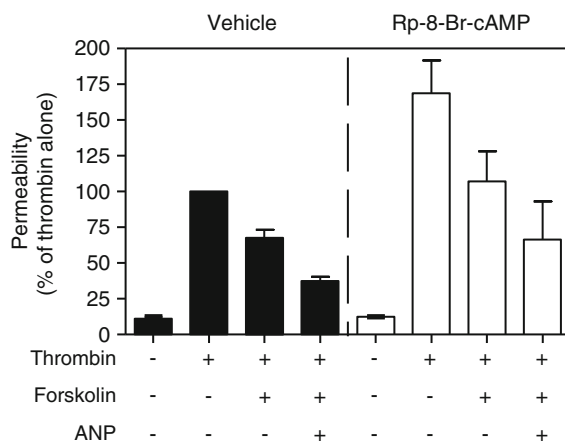


Fig. 1 PKA mediates some of the effects of cAMP on endothelial permeability. Permeability assays with thrombin, forskolin, and 0.3 nM ANP \pm 100 μ M Rp-8-Br-cAMP were performed as described previously (Surapisitchat et al. 2007). Rp-8-Br-cAMP was added 15 min prior to stimulation with thrombin, forskolin, and ANP. Data represent mean (mean \pm SEM) from two independent experiments using HUVEC isolated from two different umbilical cords

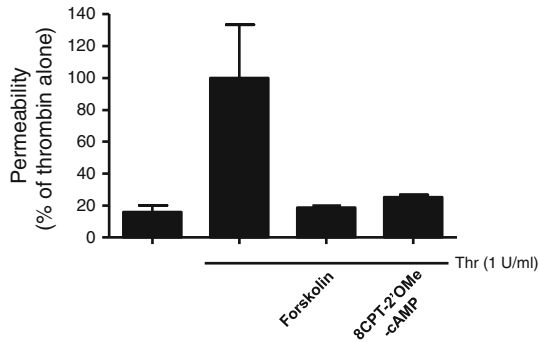


Fig. 2 Epac mediates some of the effects of cAMP on endothelial permeability. HUVEC were stimulated with thrombin, thrombin + 10 μ M forskolin, or thrombin + 100 μ M 8-CPT-2'OMe-cAMP, and permeability assays were performed as described previously (Surapisitchat et al. 2007). Data represent mean (mean \pm SEM) from two independent experiments using HUVEC isolated from two different umbilical cords

Epac to effect independent and complementary pathways to regulate endothelial barrier function (Lorenowicz et al. 2008).

While cAMP has been shown to inhibit increases in endothelial cell permeability and various mechanisms have been proposed and demonstrated, recent reports from the Steven's group demonstrate that the effects of cAMP on permeability are more complex. Sayner et al. and Prasain et al. describe results that show a barrier-disruptive effect of cAMP can be produced in the cytosol by activation of the soluble adenylyl cyclases (Prasain et al. 2009; Sayner et al. 2004, 2006). These studies suggest that cytosolic-produced cAMP decreases barrier function, but cAMP made by membrane adenylyl cyclases improves barrier function via PKA and/or Epac. The concept of compartmentalization of cyclic nucleotide signaling has been an area of increasing study of late (Fischmeister et al. 2006; Houslay 2010). It is possible that there are several cAMP "pools" that are responsible for the regulation of endothelial permeability. While the role of cAMP in regulating endothelial permeability has received substantial study, more work is needed to understand the relative contribution of the cAMP effector molecules PKA and Epac in mediating the effects of cAMP as well as the roles of compartmentalization of cAMP and its regulation in endothelial barrier function.

5 The Role of cGMP in the Regulation of Endothelial Barrier Function

The role of cGMP on endothelial barrier function is even more controversial than that of cAMP. Both barrier-enhancing and impairing effects of cGMP have been reported (Draijer et al. 1995a; van Nieuw Amerongen and van Hinsbergh 2002;

Zimmerman et al. 1990). cGMP is produced by soluble and particulate guanylyl cyclases (sGC and pGC), which are stimulated by nitric oxide or natriuretic peptides, respectively. In vitro studies have shown that NO can improve barrier function, presumably via activation of sGC and production of cGMP. Several groups demonstrated this effect via the use of exogenous NO donors such as sodium nitroprusside and DETA NONOate (Hempel et al. 1996; Suttorp et al. 1996b; Westendorp et al. 1994; Wong et al. 2004). Others have used nitric oxide synthase (NOS) inhibitors such as L-NAME to show that NO is required for maintaining endothelial barrier function (Draijer et al. 1995a; He et al. 1997b; Liu and Sundqvist 1997). In vivo, loss of NO via either genetic ablation of eNOS or treatment with L-NAME resulted in increased endothelial permeability (Predescu et al. 2005). On the other hand, several groups have shown that NO can increase endothelial permeability. For example, it was demonstrated that while NO donors and cGMP can decrease permeability in resting endothelial cells, cells stimulated with ionomycin to increase permeability showed a further increase in permeability upon addition of NO donors in vitro (Holschermann et al. 1997). Furthermore, in an ex vivo model using frog mesenteric venular microvessels, it was further demonstrated that the ability of ionomycin to increase permeability is attenuated by NOS inhibitors, suggesting a role of NO in increasing permeability (He et al. 1997a). Finally, in vivo models, including the same eNOS $-/-$ mice, were used to show that NO is involved in mediating increases of permeability under inflammatory conditions (Bucci et al. 2005; Hatakeyama et al. 2006).

Like NO, the role of natriuretic peptides in regulating endothelial permeability is also controversial. Five different groups studying endothelial permeability found that atrial natriuretic peptide (ANP) decreases endothelial permeability (Baron et al. 1989; Hempel et al. 1996; Klinger et al. 2006; Suttorp et al. 1996b; Westendorp et al. 1994). On the other hand, ANP has also been shown to increase endothelial permeability in vitro by one group (Holschermann et al. 1997). In vivo, ANP has also been shown to increase endothelial permeability (Tucker et al. 1992; Zimmerman et al. 1990). This was further supported by the finding that endothelial-specific ablation of the ANP receptor, guanylyl cyclase-A (GC-A), resulted in decreased permeability in vivo (Sabrane et al. 2005). Further complicating this issue is the questionable involvement of PKG. For example, it was reported that the barrier-enhancing effects of cGMP is PKG dependent (Moldobaeva et al. 2006). Others also found that PKG mediated the barrier regulating effects, but instead of decreasing permeability, they found that PKG increased permeability (Holschermann et al. 1997). Additionally, the barrier-enhancing effects of cGMP were reported to be PKG independent (Gupta et al. 2001). Further complicating the issue, it has been reported that the effects of cGMP are PKG dependent in endothelial cells that express PKG, while they are independent in endothelial cells that do not express PKG (Draijer et al. 1995a, b). Thus, while it is fairly clear that increased cAMP from endogenous membrane cyclases improves endothelial barrier function, the role of cGMP, whether from stimulation of sGC by NO or from pGC activated by ANP, has until recently remained unresolved.

6 Endothelial Cell Cyclic Nucleotide Phosphodiesterases

While PKA, Epac, PKG, and cyclic nucleotide-gated channels (CNG) play critical roles in mediating the effects of cAMP and cGMP in endothelial cells, the role of cyclic nucleotide phosphodiesterases (PDEs) is emerging as an equally important player in endothelial function. In addition to regulating the amplitude, duration, and compartmentalization of cyclic nucleotides, PDEs are also effector molecules of cyclic nucleotides and calcium, making them critical mediators of crosstalk between various second messengers. Endothelial cells have been shown to primarily express cGMP-stimulated PDE2, cGMP-inhibited PDE3, cAMP-specific PDE4, and cGMP-specific PDE5 (Netherton and Maurice 2005). The expression of Ca^{2+} /CaM-regulated PDE1 and the cAMP-specific PDE7A has also been described (Keravis et al. 2000; Miro et al. 2000). While these PDEs have been shown to be expressed in the endothelium, significant differences in their relative expression levels have been found in endothelial cells of different origin. The relative expression and PDE activity levels in bovine aortic endothelial cells (BAEC), human aortic endothelial cells (HAEC), human umbilical vein endothelial cells (HUVEC), and human microvascular endothelial cells (HMVEC) were determined, and large differences in the expression levels of PDE2, PDE3, PDE4, and PDE5 were found among these cells (Netherton and Maurice 2005). For example, measuring PDE activity at a substrate concentration of 1 μM cAMP, it was shown that PDE3 accounted for 15 and 36% of the cAMP activity in BAEC and HAEC, respectively, while only 7 and 6% of the cAMP activity in HUVEC and HMVEC, respectively. Differences were also seen at the protein level by immunoblot analysis. In addition to differences in PDE expression between endothelial cells of different origins, there have been differences reported in endothelial cells over time in culture. For example, early passage BAEC (4–6) expressed PDE2 and PDE5 cGMP hydrolytic activity, which were lost with passage (> 10) (Ashikaga et al. 1997). As for cAMP, PDE2 and PDE4 cAMP hydrolytic activity was found in early passage BAEC that later become predominantly PDE4 at later passages. Furthermore, there are reported differences in PDE expression in endothelial cells of different phenotypes. Confluent and resting endothelial cells take on a cobblestone-shaped morphology, while proliferating, noncontact-inhibited endothelial cells appear elongated and spindle-like. The PDE profile of BAEC has been characterized in these two different states (Keravis et al. 2000). While resting cobblestone BAEC expressed PDE2 and PDE4 cAMP hydrolytic activity, measured at a substrate concentration of 1 μM cAMP, spindle-shaped proliferating BAEC expressed increased cAMP hydrolytic activity due to increased PDE2 and PDE4 activity as well as PDE1 and PDE3 activity. cGMP-PDE activity was primarily due to PDE2 in resting cobblestone BAEC, while spindle-shaped proliferating BAEC expressed both PDE2 and PDE5. Finally, various stimuli can alter PDE expression in endothelial cells. The inflammatory cytokine, TNF- α , shown to increase endothelial expression of various adhesion molecules and decrease barrier function has also been shown to upregulate the expression of PDE2, PDE4, and PDE7 in endothelial cells

(Koga et al. 1995; Miro et al. 2000; Seybold et al. 2005). The reasons for the differences in expression of PDEs in endothelial cells of different origins and in response to different stimuli are not well understood. Many of these presumed differences may relate to differences in passage number of the cells being compared.

7 The Role of Phosphodiesterases in the Regulation of Endothelial Permeability

Since cAMP and cGMP play significant and sometimes conflicting roles in endothelial barrier function, endothelial cell PDEs may play critical roles in the regulation of endothelial permeability. The expression of cGMP-activated PDE2, cGMP-inhibited PDE3, cAMP-specific PDE4, and cGMP-specific PDE5 by endothelial cells may allow for differential regulation of barrier function by different PDEs. For example, we have recently demonstrated that cGMP elevating agents such as ANP and NO have biphasic effects on cAMP inhibition of thrombin-induced endothelial permeability (Surapisitchat et al. 2007). The reason for the biphasic actions of cGMP on cAMP inhibition of thrombin-induced endothelial permeability is due to differential regulation of PDE2 and PDE3 by cGMP. Furthermore, altering the expression levels of PDE2 and PDE3 in endothelial cells can alter the effect of cGMP on endothelial permeability as is seen upon stimulation of the cells by TNF- α (Fig. 3). These results likely explain many of the conflicting results reported in the literature concerning the actions of cGMP on endothelial permeability and suggest important

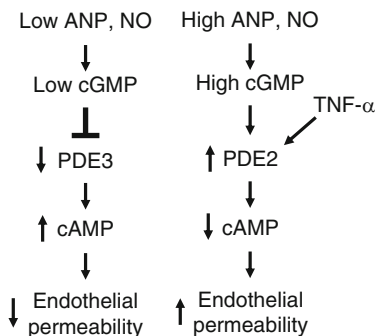


Fig. 3 Proposed model of the effect of cGMP on endothelial cell permeability. cGMP generated by NO or ANP stimulation can effect endothelial permeability in several ways. cGMP can inhibit PDE3 leading to increased cAMP in the endothelial cell and decreased permeability. cGMP can also activate PDE2 leading to decreased cAMP and increased permeability. The concentration of cGMP within the cell plays a central role in whether the dominant effect is due to PDE3 inhibition (low cGMP) or PDE2 activation (high cGMP). TNF- α can increase the amount of PDE2 expressed in the endothelial cell and thus alter the response of the cell to cGMP and permeability (from Surapisitchat et al. 2007)

roles for PDE2 and PDE3 in regulating endothelial function. Until recently, PDE4 inhibition was thought to improve endothelial barrier function via increased cAMP/PKA levels in the cell (Suttorp et al. 1993). However, other data have demonstrated that in some circumstances increased cAMP in the cytosol can cause increased endothelial permeability (Sayner et al. 2004, 2006). Recently, it was demonstrated that PDE4D4 is responsible for containing cAMP to a membrane pool, restricting cAMP access to the cytosol where it can lead to increased permeability (Creighton et al. 2008). These findings demonstrate the complexity of cyclic nucleotide signaling in the control of endothelial permeability and the critical role of PDEs in regulating this important function.

8 Crosstalk Between cGMP and cAMP: The Role of PDEs

The findings that cGMP can regulate cAMP hydrolysis by both PDE2 and PDE3 to alter cAMP levels are in agreement with previous work demonstrating a barrier-enhancing effect of cAMP and further our understanding of the regulation of cAMP in endothelial cells (Surapisitchat et al. 2007). At low concentrations, cGMP can regulate cAMP-mediated enhancement of barrier function via inhibition of PDE3, but, at higher cGMP concentrations, via activation of PDE2, it can mediate breakdown of barrier function. These results are some of the few in which the same pool of cGMP has been shown to both inhibit PDE3 and activate PDE2 to regulate the same cellular function. For example, in human atrial myocytes, cGMP can regulate L-type Ca^{2+} channel current by modulating cAMP and PKA via PDE2 and PDE3 (Vandecasteele et al. 2001). The functional effect of cGMP is concentration-dependent since the IC_{50} of cGMP for PDE3A is nearly 20-fold less than the EC_{50} for activation of PDE2 (Leroy et al. 1996; Surapisitchat et al. 2007; Yamamoto et al. 1983). This difference correlates well with normal versus pathological levels of guanylyl cyclase agonists such as ANP and NO. In healthy individuals, these agonists are relatively low, resulting in low levels of cGMP leading to inhibition of PDE3A, increased cAMP, and enhanced barrier function. Under several pathological conditions such as heart failure and inflammation, ANP and NO increase significantly leading to greatly increased cGMP levels, activation of PDE2A, decreased cAMP levels, and thus decreased barrier function. In addition under some pathological conditions, the relative expression of PDEs can be altered. For example, TNF- α , an inflammatory cytokine, induces PDE2 and decreases PDE3 expression, thereby altering the cellular responses to cGMP signaling (Seybold et al. 2005; Surapisitchat et al. 2007). Thus, PDE2 and PDE3 in the endothelium may act as sensors or switches to detect normal versus pathological concentrations of cGMP and thus regulate endothelial permeability accordingly. Furthermore, the relative expression of these PDEs controls the sensitivity of this switch to cGMP.

9 Cyclic Nucleotide Pools in Endothelial Barrier Function

Studies suggest that there may be multiple pools of cAMP and cGMP in endothelial cells regulated by PDEs (Creighton et al. 2008; Sayner and Stevens 2006; Sayner et al. 2006). This is not unexpected since it is becoming increasingly clear that cellular signaling is a highly compartmentalized phenomenon in which there are many microdomains that are differentially regulated by various proteins and stimuli (Houslay et al. 2007; Houslay 2010).

Even with our recent realization that different amounts of PDE2 and PDE3 may account for much of the variability in responses reported for cAMP and cGMP on barrier function, substantial controversy still exists. For example, in contrast to our results (Surapisitchat et al. 2007) and that of others (Adamson et al. 1998; Langelier and van Hinsbergh 1991; van Hinsbergh 1997) indicating that cAMP produced by transmembrane adenylyl cyclases decreases permeability, Sayner et al.'s (2004, 2006) work suggests that increases in a cytosolic pool of cAMP increase permeability. These authors go on to propose a model in which cAMP made by transmembrane adenylyl cyclases is maintained in a microdomain near the plasma membrane by PDE4. The idea is that if PDE4 is inhibited, cAMP can diffuse into the cytosolic compartment (Sayner et al. 2006). Recently, it has been proposed that PDE4D4 is the PDE4 isoform responsible for maintaining cAMP at the membrane (Creighton et al. 2008). This contrast, to previous results by several other groups and our own data demonstrating a barrier-enhancing effect by forskolin and PDE4 inhibition (Suttorp et al. 1996a). Our data indicate that forskolin (10 μ M) can almost completely abolish thrombin-induced permeability and that rolipram can potentiate the barrier-enhancing effects of lower forskolin concentrations (Fig. 4). This discrepancy might, however, be explained by differences in the types of endothelial cells studied, microvascular versus macrovascular (Creighton et al. 2008). In particular, microvascular cells are almost always more greatly expanded and studied at a later passage number. Nevertheless, these findings strongly suggest that there are different "pools" of cAMP regulating different aspects of barrier function.

As with cAMP, pools of cGMP regulating various distinct cellular functions have been described. This was expected with the existence of soluble and particulate guanylyl cyclases. In our studies, we found that cGMP generated from either soluble or particulate guanylyl cyclases had the same biphasic effect on permeability. It is possible though that there are still distinct pools of cGMP regulating barrier function via both PDE2 and PDE3. One possibility is that PDE2 and PDE3 are regulating the same pool of cAMP that controls permeability and two pools of cGMP regulate these PDEs, but they cannot be detected by measuring changes in permeability. For example, cGMP made by soluble guanylyl cyclases may be more effective than that of cGMP made from particulate cyclases, but this difference, a shift in the dose–response curves for the agonists, cannot be detected by measuring permeability. Complicating this further is the time variable in which levels of either cAMP or cGMP may change within a certain pool and with time. Again, many

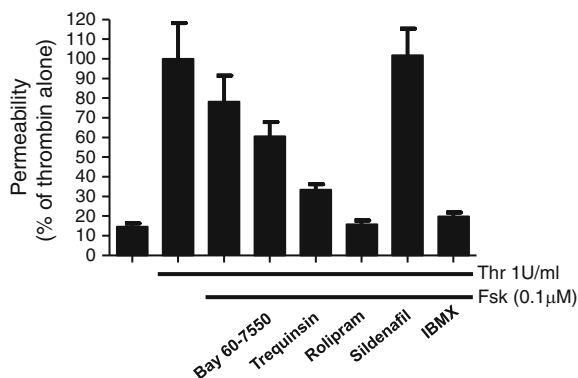


Fig. 4 Effects of PDE inhibitors on forskolin-inhibited thrombin-induced permeability. HUVEC were stimulated with vehicle, 1 U/ml thrombin (Thr), 1 U/ml thrombin and 0.1 μ M forskolin (Fsk), or thrombin, forskolin, and PDE inhibitors. Bay 60-7550 (PDE2) was used at 100 nM. Trequinsin (PDE3) was used at 30 nM. Rolipram (PDE4) was used at 10 μ M. Sildenafil (PDE5) was used at 100 nM. IBMX (nonspecific) was used at 100 μ M. All inhibitors were added 15 min prior to thrombin + forskolin stimulation. Permeability was analyzed as described previously (Surapisitchat et al. 2007). Data represent means (mean \pm SEM) from three independent experiments using HUVEC isolated from three different umbilical cords

combinations of PDEs, cyclases, effector molecules, and pools are possible. Until more sensitive techniques with increased resolution become available, an accurate description of cyclic nucleotide signaling pools in time and space will have to wait.

The findings that the inflammatory cytokine, TNF- α , can increase PDE2 and decrease PDE3A expression demonstrates that endothelial cells can be altered by various stimuli to respond to cGMP signaling under different conditions. The alteration in PDE expression in response to various stimuli is a common mechanism that many cells and tissues use to alter cyclic nucleotide signaling. For example, nitroglycerine (NTG) is used in the treatment of hypertension for its vasorelaxing effects, but its therapeutic use is limited due to the development of nitrate tolerance. In rats treated with NTG, PDE1A expression and activity is upregulated (Kim et al. 2001). Inhibition of PDE1A leads to partial restoration of smooth muscle responsiveness to nitrates. PDE1C is found to be upregulated in proliferating smooth muscle, suggesting a potential target in the treatment of atherosclerosis or restenosis after angioplasty (Rybalkin et al. 1997). PDE7 upregulation is required for T-cell activation by CD3 and CD28 (Li et al. 1999). Other examples of PDE upregulation include the upregulation of PDE1B in monocyte to macrophage differentiation and PDE5 in vascular smooth muscle in response to angiotensin II stimulation (Bender et al. 2005; Kim et al. 2005). Few examples of downregulation of PDEs have been reported to our knowledge. PDE3A expression and activity has been found to be decreased in patients with heart failure (Ding et al. 2005). In 3T3-L1 adipocytes, TNF- α decreased PDE3B expression, implicating a mechanism by which TNF- α regulates lipolysis (Rahn Landstrom et al. 2000). The findings that TNF- α can also decrease PDE3A in addition to increasing PDE2 to alter endothelial response to

cGMP signaling add to this growing list of examples in which alteration of PDE expression and activity in response to physiological stimuli can lead to altered cellular responses. Changes in PDE expression may change the “size and shape” of the normal cyclic nucleotide pool to one that is pathologic. Understanding how changes in PDE expression by pathological stimuli alter endothelial barrier function will be important for the development of treatments of disease states involving endothelial barrier dysfunction.

10 Conclusions

The last several years have been filled with new and exciting findings concerning the role of cyclic nucleotides and PDEs in the regulation of endothelial barrier function. Whereas less than ten years ago the dogma had been that cAMP improved endothelial barrier function while the role of cGMP was debatable, today the role of these two second messengers in endothelial permeability has been shown to be highly dependent on the concentration and localization of these two second messengers within the endothelial cell. PDEs have been shown to play a critical role in regulating the amplitude, duration, and localization of cAMP and cGMP in endothelial cells and thus regulate endothelial barrier function. The concept of different subcellular pools of cAMP and cGMP, controlled by PDEs, regulating different cellular functions is becoming increasingly important. Altered PDE expression in pathological states may alter these pools and thus the effects of these cyclic nucleotides. Thus, understanding the role of PDEs in regulating cyclic nucleotide signaling in normal and pathological endothelial functions may be important for the development of drugs targeting specific PDEs in order to treat diseases that are caused by endothelial dysfunction.

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References

- Adamson RH, Liu B, Fry GN, Rubin LL, Curry FE (1998) Microvascular permeability and number of tight junctions are modulated by cAMP. *Am J Physiol* 274:H1885–H1894
- Ashikaga T, Strada SJ, Thompson WJ (1997) Altered expression of cyclic nucleotide phosphodiesterase isozymes during culture of aortic endothelial cells. *Biochem Pharmacol* 54:1071–1079
- Baron DA, Lofton CE, Newman WH, Currie MG (1989) Atriopeptin inhibition of thrombin-mediated changes in the morphology and permeability of endothelial monolayers. *Proc Natl Acad Sci USA* 86:3394–3398
- Beaver AT, Ostenson CL, Wang EH, Beavo JA (2005) Selective up-regulation of PDE1B2 upon monocyte-to-macrophage differentiation. *Proc Natl Acad Sci USA* 102:497–502

- Bucci M, Roviezzo F, Posadas I, Yu J, Parente L, Sessa WC, Ignarro LJ, Cirino G (2005) Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation in vivo. *Proc Natl Acad Sci USA* 102:904–908
- Cohen AW, Hnasko R, Schubert W, Lisanti MP (2004) Role of caveolae and caveolins in health and disease. *Physiol Rev* 84:1341–1379
- Creighton J, Zhu B, Alexeyev M, Stevens T (2008) Spectrin-anchored phosphodiesterase 4D4 restricts cAMP from disrupting microtubules and inducing endothelial cell gap formation. *J Cell Sci* 121:110–119
- Cullere X, Shaw SK, Andersson L, Hirahashi J, Luscinskas FW, Mayadas TN (2005) Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. *Blood* 105:1950–1955
- Ding B, Abe J, Wei H, Huang Q, Walsh RA, Molina CA, Zhao A, Sadoshima J, Blaxall BC, Berk BC, Yan C (2005) Functional role of phosphodiesterase 3 in cardiomyocyte apoptosis: implication in heart failure. *Circulation* 111:2469–2476
- Draijer R, Atsma DE, van der Laarse A, van Hinsbergh VW (1995a) cGMP and nitric oxide modulate thrombin-induced endothelial permeability. Regulation via different pathways in human aortic and umbilical vein endothelial cells. *Circ Res* 76:199–208
- Draijer R, Vaandrager AB, Nolte C, de Jonge HR, Walter U, van Hinsbergh VW (1995b) Expression of cGMP-dependent protein kinase I and phosphorylation of its substrate, vasodilator-stimulated phosphoprotein, in human endothelial cells of different origin. *Circ Res* 77:897–905
- Fischmeister R, Castro LR, Abi-Gerges A, Rochais F, Jurevicius J, Leroy J, Vandecasteele G (2006) Compartmentation of cyclic nucleotide signaling in the heart: the role of cyclic nucleotide phosphodiesterases. *Circ Res* 99:816–828
- Fukuhara S, Sakurai A, Sano H, Yamagishi A, Somekawa S, Takakura N, Saito Y, Kangawa K, Mochizuki N (2005) Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. *Mol Cell Biol* 25:136–146
- Gloerich M, Bos JL (2010) Epac: defining a new mechanism for cAMP action. *Annu Rev Pharmacol Toxicol* 50:355–375
- Gupta MP, Ober MD, Patterson C, Al-Hassani M, Natarajan V, Hart CM (2001) Nitric oxide attenuates H₂O₂-induced endothelial barrier dysfunction: mechanisms of protection. *Am J Physiol Lung Cell Mol Physiol* 280:L116–L126
- Hatakeyama T, Pappas PJ, Hobson RW 2nd, Boric MP, Sessa WC, Duran WN (2006) Endothelial nitric oxide synthase regulates microvascular hyperpermeability in vivo. *J Physiol* 574:275–281
- He P, Liu B, Curry FE (1997a) Effect of nitric oxide synthase inhibitors on endothelial [Ca²⁺]_i and microvessel permeability. *Am J Physiol* 272:H176–H185
- He P, Zeng M, Curry FE (1997b) Effect of nitric oxide synthase inhibitors on basal microvessel permeability and endothelial cell [Ca²⁺]_i. *Am J Physiol* 273:H747–H755
- Hempel A, Noll T, Muhs A, Piper HM (1996) Functional antagonism between cAMP and cGMP on permeability of coronary endothelial monolayers. *Am J Physiol* 270:H1264–H1271
- Holschermann H, Noll T, Hempel A, Piper HM (1997) Dual role of cGMP in modulation of macromolecule permeability of aortic endothelial cells. *Am J Physiol* 272:H91–H98
- Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 35:91–100
- Houslay MD, Baillie GS, Maurice DH (2007) cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. *Circ Res* 100:950–966
- Jho D, Mehta D, Ahmmed G, Gao XP, Tiruppathi C, Broman M, Malik AB (2005) Angiopoietin-1 opposes VEGF-induced increase in endothelial permeability by inhibiting TRPC1-dependent Ca²⁺ influx. *Circ Res* 96:1282–1290
- Keravis T, Komasa N, Lugnier C (2000) Cyclic nucleotide hydrolysis in bovine aortic endothelial cells in culture: differential regulation in cobblestone and spindle phenotypes. *J Vasc Res* 37:235–249

- Kim D, Aizawa T, Wei H, Pi X, Rybalkin SD, Berk BC, Yan C (2005) Angiotensin II increases phosphodiesterase 5A expression in vascular smooth muscle cells: a mechanism by which angiotensin II antagonizes cGMP signaling. *J Mol Cell Cardiol* 38:175–184
- Kim D, Rybalkin SD, Pi X, Wang Y, Zhang C, Munzel T, Beavo JA, Berk BC, Yan C (2001) Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance. *Circulation* 104:2338–2343
- Klinger JR, Warburton R, Carino GP, Murray J, Murphy C, Napier M, Harrington EO (2006) Natriuretic peptides differentially attenuate thrombin-induced barrier dysfunction in pulmonary microvascular endothelial cells. *Exp Cell Res* 312:401–410
- Koga S, Morris S, Ogawa S, Liao H, Bilezikian JP, Chen G, Thompson WJ, Ashikaga T, Brett J, Stern DM et al (1995) TNF modulates endothelial properties by decreasing cAMP. *Am J Physiol* 268:C1104–C1113
- Komarova Y, Malik AB (2010) Regulation of endothelial permeability via paracellular and transcellular transport pathways. *Annu Rev Physiol* 72:463–493
- Komarova YA, Mehta D, Malik AB (2007) Dual regulation of endothelial junctional permeability. *Sci STKE* 2007:re8
- Kooistra MR, Corada M, Dejana E, Bos JL (2005) Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. *FEBS Lett* 579:4966–4972
- Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J (1996) Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *EMBO J* 15:510–519
- Langelier EG, van Hinsbergh VW (1991) Norepinephrine and iloprost improve barrier function of human endothelial cell monolayers: role of cAMP. *Am J Physiol* 260:C1052–C1059
- Leemhuis J, Boutillier S, Schmidt G, Meyer DK (2002) The protein kinase A inhibitor H89 acts on cell morphology by inhibiting Rho kinase. *J Pharmacol Exp Ther* 300:1000–1007
- Leroy MJ, Degerman E, Taira M, Murata T, Wang LH, Movsesian MA, Meacci E, Manganiello VC (1996) Characterization of two recombinant PDE3 (cGMP-inhibited cyclic nucleotide phosphodiesterase) isoforms, RcGIP1 and HcGIP2, expressed in NIH 3006 murine fibroblasts and Sf9 insect cells. *Biochemistry* 35:10194–10202
- Li L, Yee C, Beavo JA (1999) CD3- and CD28-dependent induction of PDE7 required for T cell activation. *Science* 283:848–851
- Liu SM, Sundqvist T (1997) Nitric oxide and cGMP regulate endothelial permeability and F-actin distribution in hydrogen peroxide-treated endothelial cells. *Exp Cell Res* 235:238–244
- Lorenowicz MJ, Fernandez-Borja M, Kooistra MR, Bos JL, Hordijk PL (2008) PKA and Epac1 regulate endothelial integrity and migration through parallel and independent pathways. *Eur J Cell Biol* 87:779–792
- Mehta D, Malik AB (2006) Signaling mechanisms regulating endothelial permeability. *Physiol Rev* 86:279–367
- Michel CC, Curry FE (1999) Microvascular permeability. *Physiol Rev* 79:703–761
- Miro X, Casacuberta JM, Gutierrez-Lopez MD, de Landazuri MO, Puigdomenech P (2000) Phosphodiesterases 4D and 7A splice variants in the response of HUVEC cells to TNF-alpha(1). *Biochem Biophys Res Commun* 274:415–421
- Moldobaeva A, Welsh-Servinsky LE, Shimoda LA, Stephens RS, Verin AD, Tudor RM, Pearse DB (2006) Role of protein kinase G in barrier-protective effects of cGMP in human pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 290:L919–L930
- Moore TM, Chetham PM, Kelly JJ, Stevens T (1998) Signal transduction and regulation of lung endothelial cell permeability. Interaction between calcium and cAMP. *Am J Physiol* 275:L203–L222
- Murray AJ (2008) Pharmacological PKA inhibition: all may not be what it seems. *Sci Signal* 1:re4
- Netherton SJ, Maurice DH (2005) Vascular endothelial cell cyclic nucleotide phosphodiesterases and regulated cell migration: implications in angiogenesis. *Mol Pharmacol* 67:263–272
- Ozaki H, Hla T, Lee MJ (2003) Sphingosine-1-phosphate signaling in endothelial activation. *J Atheroscler Thromb* 10:125–131

- Pizurki L, Zhou Z, Glynos K, Roussos C, Papapetropoulos A (2003) Angiopoietin-1 inhibits endothelial permeability, neutrophil adherence and IL-8 production. *Br J Pharmacol* 139: 329–336
- Prasain N, Alexeyev M, Balczon R, Stevens T (2009) Soluble adenylyl cyclase-dependent microtubule disassembly reveals a novel mechanism of endothelial cell retraction. *Am J Physiol Lung Cell Mol Physiol* 297:L73–L83
- Predescu D, Predescu S, Shimizu J, Miyawaki-Shimizu K, Malik AB (2005) Constitutive eNOS-derived nitric oxide is a determinant of endothelial junctional integrity. *Am J Physiol Lung Cell Mol Physiol* 289:L371–L381
- Qiao J, Holian O, Lee BS, Huang F, Zhang J, Lum H (2008) Phosphorylation of GTP dissociation inhibitor by PKA negatively regulates RhoA. *Am J Physiol Cell Physiol* 295:C1161–C1168
- Qiao J, Huang F, Lum H (2003) PKA inhibits RhoA activation: a protection mechanism against endothelial barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 284:L972–L980
- Rahn Landstrom T, Mei J, Karlsson M, Manganiello V, Degerman E (2000) Down-regulation of cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumour necrosis factor alpha and cAMP. *Biochem J* 346(Pt 2):337–343
- Rybalkin SD, Bornfeldt KE, Sonnenburg WK, Rybalkina IG, Kwak KS, Hanson K, Krebs EG, Beavo JA (1997) Calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1C) is induced in human arterial smooth muscle cells of the synthetic, proliferative phenotype. *J Clin Invest* 100:2611–2621
- Sabrane K, Kruse MN, Fabritz L, Zetsche B, Mitko D, Skryabin BV, Zwiener M, Baba HA, Yanagisawa M, Kuhn M (2005) Vascular endothelium is critically involved in the hypotensive and hypovolemic actions of atrial natriuretic peptide. *J Clin Invest* 115:1666–1674
- Satchell SC, Anderson KL, Mathieson PW (2004) Angiopoietin 1 and vascular endothelial growth factor modulate human glomerular endothelial cell barrier properties. *J Am Soc Nephrol* 15:566–574
- Sayner S, Stevens T (2006) Soluble adenylate cyclase reveals the significance of compartmentalized cAMP on endothelial cell barrier function. *Biochem Soc Trans* 34:492–494
- Sayner SL, Alexeyev M, Dessauer CW, Stevens T (2006) Soluble adenylyl cyclase reveals the significance of cAMP compartmentation on pulmonary microvascular endothelial cell barrier. *Circ Res* 98:675–681
- Sayner SL, Frank DW, King J, Chen H, VandeWaa J, Stevens T (2004) Paradoxical cAMP-induced lung endothelial hyperpermeability revealed by *Pseudomonas aeruginosa* ExoY. *Circ Res* 95:196–203
- Seybold J, Thomas D, Witzenth M, Boral S, Hocke AC, Burger A, Hatzelmann A, Tenor H, Schudt C, Krull M, Schutte H, Hippenstiel S, Suttorp N (2005) Tumor necrosis factor-alpha-dependent expression of phosphodiesterase 2: role in endothelial hyperpermeability. *Blood* 105:3569–3576
- Surapitschat J, Jeon KI, Yan C, Beavo JA (2007) Differential regulation of endothelial cell permeability by cGMP via phosphodiesterases 2 and 3. *Circ Res* 101:811–818
- Suttorp N, Ehreiser P, Hippenstiel S, Fuhrmann M, Krull M, Tenor H, Schudt C (1996a) Hyperpermeability of pulmonary endothelial monolayer: protective role of phosphodiesterase isoenzymes 3 and 4. *Lung* 174:181–194
- Suttorp N, Hippenstiel S, Fuhrmann M, Krull M, Podzuweit T (1996b) Role of nitric oxide and phosphodiesterase isoenzyme II for reduction of endothelial hyperpermeability. *Am J Physiol* 270:C778–C785
- Suttorp N, Weber U, Welsch T, Schudt C (1993) Role of phosphodiesterases in the regulation of endothelial permeability in vitro. *J Clin Invest* 91:1421–1428
- Takuwa Y (2002) Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. *Biochim Biophys Acta* 1582:112–120
- Tsigkos S, Koutsilieris M, Papapetropoulos A (2003) Angiopoietins in angiogenesis and beyond. *Expert Opin Investig Drugs* 12:933–941

- Tucker VL, Simanonok KE, Renkin EM (1992) Tissue-specific effects of physiological ANP infusion on blood-tissue albumin transport. *Am J Physiol* 263:R945–R953
- van Hinsbergh WM (1997) Endothelial permeability for macromolecules. Mechanistic aspects of pathophysiological modulation. *Arterioscler Thromb Vasc Biol* 17:1018–1023
- van Nieuw Amerongen GP, Loomans DS, van Hinsbergh VW (2001) Mechanisms involved in endothelial hyperpermeability and endothelial barrier stabilization. In: Catravas JD (ed) *Vascular endothelium: source and target of inflammatory mediators*. IOS Press, Amsterdam
- van Nieuw Amerongen GP, van Hinsbergh VW (2002) Targets for pharmacological intervention of endothelial hyperpermeability and barrier function. *Vascul Pharmacol* 39:257–272
- Vandecasteele G, Verde I, Rucker-Martin C, Donzeau-Gouge P, Fischmeister R (2001) Cyclic GMP regulation of the L-type Ca(2+) channel current in human atrial myocytes. *J Physiol* 533:329–340
- Verin AD, Gilbert-McClain LI, Patterson CE, Garcia JG (1998) Biochemical regulation of the nonmuscle myosin light chain kinase isoform in bovine endothelium. *Am J Respir Cell Mol Biol* 19:767–776
- Westendorp RG, Draijer R, Meinders AE, van Hinsbergh VW (1994) Cyclic-GMP-mediated decrease in permeability of human umbilical and pulmonary artery endothelial cell monolayers. *J Vasc Res* 31:42–51
- Witthen ES, Worthylake RA, Kelly P, Casey PJ, Quilliam LA, Burrige K (2005) Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. *J Biol Chem* 280:11675–11682
- Wong D, Dorovini-Zis K, Vincent SR (2004) Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood–brain barrier. *Exp Neurol* 190:446–455
- Yamamoto T, Manganiello VC, Vaughan M (1983) Purification and characterization of cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from calf liver. Effects of divalent cations on activity. *J Biol Chem* 258:12526–12533
- Yatomi Y, Ruan F, Hakomori S, Igarashi Y (1995) Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood* 86:193–202
- Zimmerman RS, Trippodo NC, MacPhee AA, Martinez AJ, Barbee RW (1990) High-dose atrial natriuretic factor enhances albumin escape from the systemic but not the pulmonary circulation. *Circ Res* 67:461–468

Phosphodiesterases as Targets for Intermittent Claudication

Yongge Liu, Yasmin Shakur, and Junichi Kambayashi

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Abstract Intermittent claudication (IC) is one of the most frequent forms of lower extremity peripheral arterial disease (PAD) and is most commonly caused by arterial atherosclerosis. Its clinical manifestation includes fatigue, discomfort, or pain occurring in limb muscles due to exercise-induced ischemia, thus limiting the ability of IC patients to walk and exercise. In addition to lifestyle changes (diet, exercise, and smoking cessation), pharmacological treatments are needed. Pathologically, atherosclerotic lesions cause a mismatch in oxygen supply and metabolic demand in the leg muscles during walking/exercise. This subjects the muscles to repeated ischemia and reperfusion injury that can alter structure and oxidative metabolism, resulting in

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insufficient utilization of oxygen supply. Despite extensive research efforts, cilostazol and pentoxifylline are the only drugs indicated for relieving the symptoms of IC, with cilostazol demonstrating significant improvement in walking distance and quality of life in these patients. Originally developed as a PDE3 inhibitor, cilostazol was later found to have several other pharmacological actions, and its success has been attributed to its multifactorial actions on platelets, endothelium, smooth muscle, and lipid profiles. Using cilostazol as an example, we discuss the rationales and pitfalls of targeting PDEs in IC, and potential strategies for the development of new and more effective pharmacological treatments.

Keywords Adenosine · Atherosclerosis · Cilostazol · Intermittent claudication · Peripheral arterial disease · Phosphodiesterase

Abbreviation List

ABI	Ankle-brachial index
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
GC	Guanylyl cyclase
Gi	Inhibitory G-protein
GP	Glycoprotein
Gs	Stimulatory G-protein
IC	Intermittent claudication
NO	Nitric oxide
PAD	Peripheral arterial disease
PDE	Phosphodiesterase
TXA ₂	Thromboxane A ₂
VSMC	Vascular smooth muscle cell

1 Overview of Intermittent Claudication

Peripheral arterial disease (PAD) is a common manifestation of systemic atherosclerosis. Age, cigarette smoking, and diabetes mellitus are the major risk factors, along with hyperlipidemia, hypertension, and hyperhomocysteinemia. Many people with this disease are asymptomatic, but about one third of patients with PAD of the lower extremities have intermittent claudication (IC) (Hiatt 2001). IC limits the ability to walk and perform daily activities and severely affects the quality of life of patients. The severity of claudication progresses slowly, and patients may eventually develop critical limb ischemia, with substantial risk of limb loss.

1.1 Pathophysiology of IC

Typical symptoms of IC are leg-muscle fatigue, discomfort, or pain on exertion that are relieved with rest. The underlying pathophysiology involves a limitation in blood supply during walking/exercise leading to repeated ischemia-reperfusion injury and dysfunctional skeletal muscle metabolism (Fig. 1). Individuals with IC have sufficient blood flow to the leg at rest so that limb ischemic symptoms are absent. However, during walking, the oxygen demand in the leg skeletal muscle is increased and in atherosclerotic arteries such demand cannot be met by increasing blood flow. Continued muscle performance must then rely on oxygen-independent ATP production from phosphocreatine hydrolysis and glycolysis (Greenhaff et al. 2004). Ischemia and the accumulation of metabolic byproducts can lead to the rapid onset of muscle pain and fatigue.

In addition to reduced blood supply, persistent pathophysiological changes occur in the skeletal muscle of IC patients as a consequence of repeated episodes of ischemia and reperfusion injury. Histologically, patients with PAD have a lower portion of type I muscle fiber and fewer capillaries per muscle fiber (Askew et al. 2005). Skeletal muscle in these patients also demonstrates defective mitochondrial function, including the accumulation of acyl-coenzyme A intermediate and acylcarnitine, implying

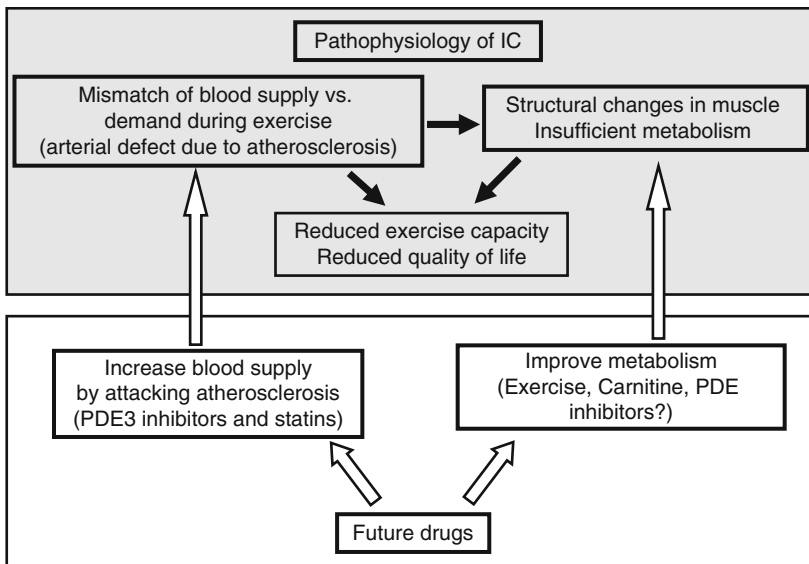


Fig. 1 Pathology of IC (gray box, top). Due to atherosclerosis, arteries are significantly narrowed, resulting in reduced perfusion to the leg muscles during walking/exercise. Repeated ischemia and reperfusion of the muscle causes pathological changes to the structure of the muscle and its energy metabolism. The manifestation of these changes results in the symptoms of intermittent claudication (IC) and reduced quality of life. Future drug development may be directed toward improvement of blood supply and normalization of muscle metabolism (white box, bottom)

that acyl-CoA is not being efficiently oxidized, which can lead to reduced energy production in the affected muscle (Hiatt 2004). Thus, defects in both the blood supply and the muscle metabolism may contribute to symptoms and reduced exercise performance in IC patients.

1.2 Diagnosis

Diagnosis involves initial screening using the ankle-brachial index (ABI). ABI is the ratio of systolic blood pressure in the ankle to blood pressure in the arm (Hirsch et al. 2006). The severity of PAD can be classified using the Fontaine Staging System. IC is categorized as stage 2 when the ABI is in the range of 0.4–0.9. The gold standard for diagnosis and evaluation of IC is treadmill (exercise) testing. For the best evaluation of a treatment regimen, two walking distances are valuable: the initial claudication distance, i.e., the distance at which the patient first experiences pain with exertion, and the absolute claudication distance, i.e., the distance at which the patient can no longer ambulate (Mohler et al. 2001).

1.3 Management

There are generally two objectives for treatment of IC. The first is the overall reduction of cardiovascular morbidity and mortality. PAD is an important manifestation of systemic atherosclerosis, and thus a diagnosis often indicates an increased atherosclerotic risk in other vascular areas. The severity of PAD is closely associated with the risk of myocardial infarction, ischemic stroke, and death from vascular causes (Hirsch et al. 2006). Thus, treatments in general should include aggressive risk factor modification. Because atherosclerosis is the hallmark of PAD and IC, lifestyle changes, such as smoking cessation and exercising, that can alleviate and reduce the progression of atherosclerosis can often reduce cardiovascular risk. Pharmacological treatments that improve lipid profiles, inhibit platelet activation, and reduce blood pressure have also been shown to offer cardio- and cerebral protection (Hankey et al. 2006; Hirsch et al. 2006).

The second objective involves treatment strategies specific to the clinical manifestation of IC: reduced walking capacity and quality of life. To this end, exercise is the most established therapy for relief of symptoms associated with IC. The benefit of exercise is most likely related to an improvement in muscle microcirculation, endothelial function, and muscle metabolism, rather than an increase in blood supply in the large vessels (Stewart et al. 2002). Other beneficial effects of exercise include lowering of lipids, glucose, and blood pressure (Hirsch et al. 2006). However, pharmacotherapies for risk reduction, such as statins, antiplatelet, and blood pressure control have not demonstrated a clinically meaningful improvement in walking distance and exercise capacity (Hiatt 2001). Currently, there are only

two approved pharmacological agents in the USA that are indicated for relief of IC symptoms and increased walking distance: cilostazol and pentoxifylline, with cilostazol demonstrating superiority over pentoxifylline. The mechanisms underlying their effects are not entirely clear, but both compounds have the property of inhibiting phosphodiesterases (PDEs), albeit with different isoform selectivity and potency. In this chapter, we use cilostazol as an example to discuss the potential utilization and limitation of PDE inhibitors in the treatment of IC. We hope that this will serve as a primer for future drug development in IC, and PAD in general, in the search for improved drugs that can both reduce cardiovascular risk and improve exercise capacity and quality of life.

2 Cells Contributing to Atherosclerosis

Since the root cause of PAD, including IC, is atherosclerosis, it is important to understand the pathology of this process as summarized in Fig. 2. The major players include endothelium, macrophages, platelets, lipid, and smooth muscle cells.

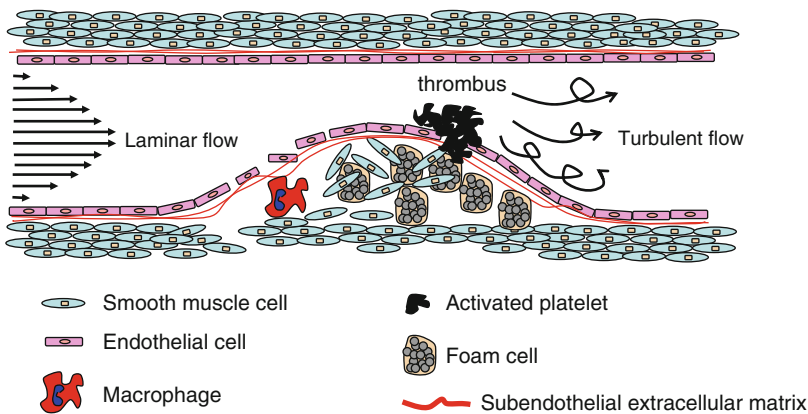


Fig. 2 Major players of arterial atherosclerosis. Endothelial damage is likely to be the initiating event in plaque formation arising as a result of chemical forces such as oxidized low-density lipoprotein, which can increase oxygen free radical production (Galle et al. 2000) and induce endothelial cell apoptosis (Dimmeler et al. 1997). Damaged endothelium leads to the activation and attachment of platelets, monocytes, and T lymphocytes at the site of the injury. Inflammatory cytokines and growth factors, released by these cells as well as smooth muscle cells of the vascular wall, promote the migration of monocytes and leukocytes into the subendothelial space. Macrophages, generated by differentiation of monocytes, then ingest oxidized lipid to form foam cells and fatty streaks. Proliferating VSMCs along with the continuing influx and propagation of macrophages converts fatty streaks to more advanced lesions and ultimately to a fibrous plaque that protrudes into the arterial lumen. Arterial atherosclerosis also produces favorable conditions for thrombosis to occur. Platelets may be attracted to the dysfunctional endothelium expressing high levels of adhesion molecules or directly interact with subendothelial thrombogenic extracellular matrix proteins. Hemodynamics also favor the formation of thrombi downstream of the protruding plaque due to the low shear and turbulent flow (Nesbitt et al. 2009)

Atherosclerosis is a progressive disease that evolves over a lifetime and ultimately results in a fibrous plaque that protrudes into the arterial lumen (Madamanchi et al. 2005). An atherosclerotic plaque poses several problems to the patient. Significant narrowing of the artery can reduce downstream blood flow, especially during elevated demand as occurs in walking and exercising. Maximal vasodilation may be unable to compensate, resulting in a mismatch in oxygen supply and metabolic demand, leading to ischemia and accumulation of metabolic byproducts that can cause the symptoms of IC. In addition, atherosclerotic vasculature may have reduced relaxation capacity (Barton and Haudenschild 2001; Wang et al. 2002) further exacerbating the situation. The narrowing of the vessel also increases the risk of thrombus development in low-shear zones downstream of the plaque (Nesbitt et al. 2009). Finally, an unstable plaque may rupture and the resulting emboli may occlude arteries in other vascular sites. The rupture also exposes highly prothrombogenic extracellular matrix components, including collagen, at the site, and partial or complete thrombotic occlusion may occur.

3 PDE Expression and Effects of PDE Inhibition in Cells Involved in Atherosclerosis

Cyclic nucleotides including cAMP and cGMP are major regulators of cellular function in the cardiovascular system and likely influence atherosclerotic pathways. Cyclic AMP and cGMP are produced by adenylyl and guanylyl cyclases (AC and GC), respectively, and hydrolyzed by PDEs. The pharmacological inhibition of PDEs increases intracellular levels of cyclic nucleotides. PDEs have been classified into 11 genetically distinct families displaying different biochemical and pharmacological properties (Maurice et al. 2003; Bender and Beavo 2006; Conti and Beavo 2007).

In this section, we discuss the expression of PDEs in platelets, vascular smooth muscle cells (VSMCs), endothelium, and adipocytes, all major contributors to atherosclerosis and thus potential mediators of PAD, including IC. PDE expression in macrophages and the effects of PDE inhibitors on inflammatory pathways are discussed in Page and Spina (2011).

3.1 Platelets

Platelets play an important role in normal hemostasis. They respond to leaks in the circulation and, acting in synergy with the coagulation system, form a thrombus to stop the bleeding. However, under pathophysiological conditions, activation of platelets (including adhesion, degranulation, and aggregation) and generation of thrombi may reduce/block blood flow causing ischemia. Thrombosis is often

caused by the exposure of collagen, the most thrombogenic constituent of the subendothelial matrix. The interaction of platelet glycoprotein GPIb-V-IX receptor complex via von Willebrand factor with exposed collagen initiates reversible recruitment of platelets to the injured site. Subsequently, binding of platelet glycoprotein (GP) VI and the inside-out activation of integrins, such as GPIa/IIa and GPIIb/IIIa, results in the firm adhesion of platelets to the injured vessel wall. Thereafter, a platelet plug is formed by the recruitment of additional platelets from the circulation, by linking through GPIIb/IIIa and the release of the secondary mediators, adenosine diphosphate (ADP) and thromboxane A₂ (TXA₂). These agonists, together with locally produced thrombin, contribute to platelet activation by stimulating receptors that couple to heterotrimeric G proteins (G_q and G_i). The end result is the elevation of intracellular calcium, leading to platelet shape change and to a further increase and production of local mediators that foster activation. This, in turn, serves as a positive-feedback mechanism to amplify the initial signals, ensuring the rapid activation and recruitment of platelets in the growing thrombus (Jackson et al. 2009; Nieswandt and Watson 2003).

Many platelet antagonists that are currently in clinical use block only one activation pathway: for example, ADP-receptor antagonists inhibit the ADP-mediated pathway, while aspirin blocks the formation and secretion of TXA₂. However, elevation of intracellular cyclic nucleotides interferes with all known platelet activation pathways, including adhesion, degranulation, aggregation, and even downregulation of proinflammatory platelet surface molecules, an effect very important for the prevention and development of atherosclerotic lesions (Schwarz et al. 2001), making cyclic nucleotides attractive targets for inhibiting platelet activation.

In platelets, three types of PDEs (PDE2, 3, and 5) have been detected. Among these, inhibitors of PDE3 have been shown to be the most potent in inhibiting platelet activation. These inhibitors include milrinone, cilostamide, enoxamide, cilostazol, and NT-702 (Ishiwata et al. 2007). Thus, there has been much interest in PDE3 inhibitors for blocking platelet activation. There are two subtypes of PDE3, PDE3A, and 3B, with 3A being the predominant subtype in platelets (Sun et al. 2007). Current PDE3 inhibitors inhibit both PDE3A and 3B. The effects of cilostazol on platelets are discussed in more detail in Sect. 4.1. PDE2 inhibitors have been reported to have no effect on platelet activation (Dunkern and Hatzelmann 2005; Zhang and Colman 2007). Although PDE5 inhibitors are able to potentiate nitric oxide donor-mediated inhibition of platelet activation (Gudmundsdottir et al. 2005; Schmidt et al. 2001; Wilson et al. 2008), contradictory findings have been reported on the effect of PDE5 inhibitors alone, with some studies reporting inhibition (Chiu et al. 1997; Gudmundsdottir et al. 2005) and others reporting a lack of inhibition of platelet activation (Dunkel et al. 2007; Schmidt et al. 2001; Wilson et al. 2008). However, in those studies that demonstrating a PDE5 inhibitor-mediated suppression of platelet activation, it is not clear whether this effect is due directly to cGMP-dependent protein phosphorylation or to an indirect effect involving cGMP-mediated inhibition of PDE3 via cGMP's competition for cAMP hydrolysis at the active site of PDE3 (Maurice 2005; Zaccolo and Movsesian 2007).

The latter elevates cAMP indirectly, and could explain the observed inhibition of platelet activation by PDE5 inhibitors.

3.2 *Vascular Smooth Muscle Cells*

VSMCs have been defined to exist in two distinct phenotypes: (1) the contractile/quiescent state, which functions primarily to regulate blood vessel size in healthy undamaged blood vessels, and (2) the proliferative/migratory state, which is associated mainly with the development of pathological conditions (Maurice et al. 2003), such as the development of plaque and restenosis (renarrowing of an artery previously treated by angioplasty or stenting). Both cAMP and cGMP can produce relaxation of VSMCs. While PDEs 1, 2, 3, 4, and 5 are reportedly expressed in VSMCs (Maurice et al. 2003), only inhibitors of PDE3 and 5 have demonstrated significant effects on relaxation. PDE3 inhibitors produce broad vasodilatory effects in vitro and in vivo. Vasorelaxation by PDE5 inhibitors is more profound in the penile corpus cavernosum and the pulmonary arteries. PDE3 inhibitors, in particular cilostazol, reduce VSMC proliferation in vitro and significantly slow the restenosis process in animals and patients (see Sect. 4.1.3.1). Thus, PDE3 inhibitors can modulate both phenotypes of VSMCs by relaxing the contractile cells and inhibiting the proliferative cells.

3.3 *Endothelium*

The expression of PDEs in vascular endothelium varies depending in different vascular beds, with PDEs 2, 3, 4, and 5 as the most often detected (Keravis et al. 2007; Netherton and Maurice 2005). The expression levels are also influenced by the phenotypic state (quiescent as in the healthy state, and proliferative as in the angiogenic state) (Keravis et al. 2007), and in vitro culture conditions (Ashikaga et al. 1997). Elevation of cAMP via inhibition of PDE4 inhibits cell proliferation, migration, and cell cycle progression in vascular endothelial cells (Keravis et al. 2007; Netherton and Maurice 2005). Similar effects have also been observed using PDE5 inhibitors (Zhu et al. 2005). In addition, increases in cAMP via inhibition of either PDE3 (Yang et al. 2006; Torii et al. 2007) or PDE4 (Sanz et al. 2007; Thompson et al. 2002) resulted in decreased leukocyte–endothelium interaction and a reduction of microvascular permeability. Thus, PDE3 and PDE4 inhibitors may offer protection for the endothelium under ischemia or inflammatory stress.

3.4 *Adipocytes*

PDE3B is the major PDE isoform expressed in adipocytes, and its inhibition increases lipolysis (Snyder 1999; Snyder et al. 2005). Increased lipolysis was

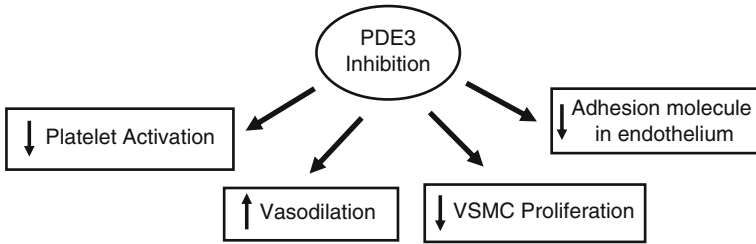


Fig. 3 Major effects from PDE3 inhibition in the vascular system. Due to the wide expression of PDE3 in the cardiovascular system, PDE3 inhibitors exert a plethora of beneficial effects targeting different cells involved in the atherosclerosis pathway

observed in both milrinone-treated rats (Cheung et al. 2003) and PDE3B-null mice, and consequently, fatty acid release was increased in these mice (Choi et al. 2006). In contrast, cilostazol reduces plasma triglycerides (Elam et al. 1998; Nakamura et al. 2003), suggesting that this effect may be mediated by a PDE3-independent mechanism.

Figure 3 summarizes the effects of PDE3 inhibitors on cells involved in atherosclerosis. As discussed above, these effects may mitigate atherosclerosis-associated diseases such as IC.

4 Pharmacotherapy for Claudication

Increasing walking distance by drug therapy has proved to be very challenging. As summarized in the ACC/AHA 2005 practice guidelines (Hirsch et al. 2006), vasodilators, including papaverine, prostaglandins (beraprost and iloprost), and calcium-channel blockers, have not demonstrated clinical efficacy. Nutritional supplements that exhibit vasodilatory or antioxidant properties, e.g., ginkgo biloba, vitamin E, and L-arginine, have also failed in large clinical trials. Antiplatelet agents, including aspirin and clopidogrel, which may reduce cardiovascular events, do not increase walking distance. L-carnitine and propionyl-L-carnitine, which were designed to facilitate the transfer of acyl groups from CoA into mitochondria and improve energy utilization, were investigated in several small clinical studies (Barker et al. 2001; Dal et al. 1999; Hiatt et al. 2001; Hiatt 2004). Although some improvement in walking distance was observed, large clinical studies are lacking, and neither drug is approved for this indication.

Currently, only cilostazol and pentoxifylline (for chemical structures, see Fig. 4) are approved to relieve symptoms associated with IC and increase walking distance. A head-to-head comparison between cilostazol and pentoxifylline has demonstrated the superior efficacy of cilostazol (Dawson et al. 2000). While both compounds are PDE inhibitors, cilostazol shows greater selectivity for PDE3 than pentoxifylline (Table 1). Both cilostazol and pentoxifylline inhibit adenosine

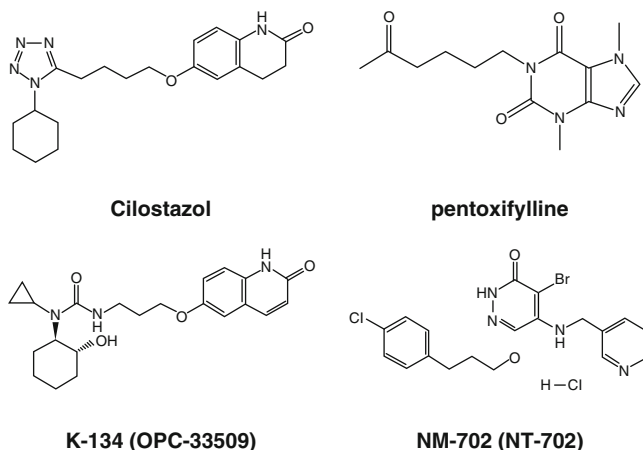


Fig. 4 Chemical structures of PDE inhibitors that have been evaluated for the treatment of intermittent claudication

Table 1 IC_{50} (μ M) of PDE inhibitors developed for use in treatment of intermittent claudication

PDE	1	2	3A	3B	4	5
Cilostazol	>100 (Sudo et al. 2000)	45.2 (Sudo et al. 2000)	0.2 (Sudo et al. 2000)	0.38 (Sudo et al. 2000)	88.0 (Sudo et al. 2000)	4.4 (Sudo et al. 2000)
Pentoxifylline ^a	>100 ^a		>100 ^a		30 ~ 100 ^a	44 ^a
NT-702 (NM-702)	3,340 (Ishiwata et al. 2007)	137 (Ishiwata et al. 2007)	0.179 (Ishiwata et al. 2007)	0.260 (Ishiwata et al. 2007)	1,240 (Ishiwata et al. 2007)	87.2 (Ishiwata et al. 2007)
K-134 (OPC-33509)	>300 (Sudo et al. 2000)	>300 (Sudo et al. 2000)	0.10 (Sudo et al. 2000)	0.28 (Sudo et al. 2000)	>300 (Sudo et al. 2000)	12.1 (Sudo et al. 2000)

Values were obtained using recombinant human PDEs expressed in Sf9 insect cells. For details, please see corresponding references. Also, isoform-specific values for particular PDE families are not listed, except for PDE3. In general, these inhibitors do not discriminate between different isoforms

^aValues for pentoxifylline were obtained from Caliper Life Sciences website <http://www.caliperls.com/assets/022/8270.pdf> (last access on Dec 2009)

uptake, but cilostazol is a much more potent inhibitor [IC_{50} : 3–5 μ M (Liu et al. 2000) vs. >100 μ M for pentoxifylline (de la Cruz et al. 1993)].

4.1 The Success Story of Cilostazol

4.1.1 Clinical Studies Leading to the Approval of Cilostazol for Relief of IC Symptoms

In the early 1990s, Otsuka began to evaluate the effect of cilostazol to increase walking distance in IC patients. Results from clinical trials in the USA in patients with moderate-to-severe IC demonstrated that cilostazol significantly increased

walking distances on the treadmill and improved quality of life in comparison with placebo (see review by (Kambayashi and Liu 2007)). This led to the approval of cilostazol in the USA, and subsequently several EU countries, for the relief of symptoms of IC and improvement in walking distance.

4.1.2 Mechanisms of Cilostazol

Cilostazol is a 2-oxo-quinoline derivative that was synthesized in 1983 by Otsuka Pharmaceutical Company, Tokushima, Japan (Nishi et al. 1985) as a PDE3 inhibitor. The biological effects of cilostazol that may potentially be relevant to the treatment of IC are discussed below. However, the exact mechanisms underlying the observed benefits have not yet been fully elucidated.

Antiplatelet and Vasodilatory Effects

The antiplatelet effect of cilostazol has been reviewed extensively elsewhere (Goto 2005; Kambayashi and Liu 2007). Cyclic AMP-mediated effects inhibit the common pathways of platelet activation, and so inhibition by cilostazol has a broad-spectrum of effects irrespective of the platelet activator that is used: ADP, collagen, epinephrine, arachidonic acid, thrombin receptor activating peptide, and shear stress-induced platelet activation.

Compared to the substantial research that has been conducted on the antiplatelet effects of cilostazol, its effects on vasodilation have been less well investigated. Studies have shown that cilostazol has broad vasodilatory effects, but with variable magnitude in different vascular beds. Vasodilation was reported to be most potent in femoral arteries (Kimura et al. 1985). In a recent investigation, we reported that cilostazol was able to increase tissue blood flow in working leg muscle, while having no effect on resting muscle (Fong et al. 2010).

Additional Mechanism: Inhibition of Adenosine Uptake

In addition to PDE3 inhibition, we found that cilostazol inhibits adenosine uptake and increases interstitial and circulatory adenosine levels in ischemic tissue (Liu et al. 2000; Manickavasagam et al. 2007; Sun et al. 2002; Wang et al. 2001). A later study confirmed this observation by showing that cilostazol can increase tissue adenosine levels during ischemia (Manickavasagam et al. 2007). Adenosine possesses a wide range of biological activities that are mediated via the activation of G-protein-coupled adenosine receptors. In platelets and VSMCs, G_s-coupled adenosine A₂ receptors dominate, and their stimulation increases intracellular cAMP. Thus, cilostazol can both increase the production and inhibit the breakdown of cAMP in platelets and VSMCs resulting in a sustained increase in the level of intracellular cAMP (Kambayashi et al. 2003; Kambayashi and Liu 2007; Liu et al. 2001).

In contrast, cardiac myocytes express mainly Gi-coupled adenosine A₁ receptors, activation of which reduces cAMP generation, thus limiting the inhibition of PDE3 in the heart. This unique feature may contribute to the safety profile of cilostazol (see Sect. 4.1.5).

4.1.3 Other Effects

In addition to its vasodilatory and inhibitory effects on platelet activation, other beneficial effects of cilostazol, resulting from PDE3-dependent and -independent actions, are discussed below.

Reduction of Restenosis

Proliferation and migration of VSMCs contribute to the growth of atherosclerotic lesions and narrowing of the vascular lumen. In addition to numerous small controlled studies (see Kambayashi and Liu 2007), one large clinical study (The Cilostazol for RESTenosis (CREST) trial) clearly demonstrated a reduction in restenosis when cilostazol was administered (Douglas et al. 2005). PDE3 inhibition and increases in intracellular cAMP levels are likely the causes of inhibition of restenosis since several studies have demonstrated PDE3 inhibitors can inhibit VSMC proliferation (Indolfi et al. 2000; Osinski and Schror 2000; Zhao et al. 2008). However, it remains to be determined whether the mechanism of cilostazol's action actually slows the progression of atherosclerosis or inhibits VSMC proliferation in IC patients.

Effects on Endothelium

Two studies have shown that cilostazol can increase PGE₁ (Igawa et al. 1990) and PGI₂ (Ito et al. 2010) release from vascular endothelial cells. These prostaglandins can stimulate Gs to increase cAMP production and are thus likely to synergize with PDE3 inhibitors as demonstrated by Igawa's study (Igawa et al. 1990) on inhibition of platelet activation. In addition, cilostazol has been shown to reduce both high-glucose-mediated expression of adhesion molecules in endothelium (Omi et al. 2004) and platelet-derived growth factor production (Mizutani et al. 1996). Cilostazol can also inhibit leukocyte adhesion to endothelium (Mori et al. 2007; Omi et al. 2004). Protection of the endothelium by cilostazol has also been observed following injury induced by ischemia (Iwama et al. 2007) or lipopolysaccharide (Kim et al. 2002). It remains to be determined whether the protective effects of cilostazol on the endothelium are due to PDE3-dependent or independent actions, or both. One study has confirmed the involvement of cAMP-mediated signaling pathways in the modulation of endothelial cells adhesive interactions by cilostazol (Mori et al. 2007). Since endothelium plays an important role in atherosclerosis and

thrombosis, the effect of cilostazol to protect against endothelial damage may contribute substantially to its overall efficacy.

Effects on Lipid Metabolism

Hyperlipidemia is one of the risk factors for PAD, and it is interesting that cilostazol has favorable effects on the plasma lipid profile. Following 12 weeks of cilostazol treatment, plasma levels of triglycerides were decreased and high-density lipoprotein cholesterol and apolipoprotein A₁ were increased while remnant lipoprotein concentrations were decreased in IC patients (Elam et al. 1998; Wang et al. 2003). Furthermore, cilostazol seems to be particularly effective in improving lipid metabolism in type 2 diabetes patients (Ikewaki et al. 2002; Ishikawa et al. 1997; Mishima et al. 2000; Takayoshi et al. 2001; Tamai et al. 1992; Watanabe et al. 1996). However, there is insufficient information to determine whether these beneficial effects are the result of cAMP-mediated mechanisms.

Protection Against Ischemia/Reperfusion Injury

Leg muscles in IC patients are expected to experience periods of ischemia during walking/exercise. Thus, treatments that can reduce ischemia-induced tissue injury may be beneficial. Preclinical studies have shown such protection by cilostazol in cardiac muscle. Cilostazol reduced myocardial infarct size after ischemia and reperfusion (Fukasawa et al. 2008; Manickavasagam et al. 2007). However, this protection may not be related to PDE3 inhibition, as milrinone, a PDE3 inhibitor, demonstrated no protection (Fukasawa et al. 2008). Reduction of cerebral infarction by cilostazol after stroke has also been reported in numerous studies (Choi et al. 2002; Honda et al. 2006; Ito et al. 2010; Lee et al. 2004; Wakida et al. 2006; Yuzawa et al. 2008). However, the data also pointed to PDE3-independent mechanisms being involved in the neuroprotection by cilostazol (Lee et al. 2004; Wakida et al. 2006). These mechanisms include an increase in adenosine levels (Manickavasagam et al. 2007), activation of calcium-activated potassium channels (Fukasawa et al. 2008; Lee et al. 2004) and metallothionein induction (Wakida et al. 2006).

Nitric Oxide

Several studies have reported the ability of cilostazol to increase NO release from endothelial cells (Nakamura et al. 2001), through PDE3 inhibition (Hashimoto et al. 2006). Similar effects have also been reported in other cell types and in vivo (Ikeda et al. 1996; Inada et al. 1999; Manickavasagam et al. 2007). This may be an important part of cilostazol's action, since a reduction in NO availability impairs vascular relaxation and accelerates the progression of atherosclerosis (Barton and Haudenschild 2001; Loscalzo 2001; Traupe et al. 2003). NO activates the soluble (NO-sensitive) GC, thus increasing production of cGMP. Another possible

mechanism resulting in elevation of cGMP by cilostazol is via inhibition of PDE5. Inhibition of PDE5 expressed in insect cells (IC_{50} of 4.4 μ M) has been reported for cilostazol (Sudo et al. 2000). However, we did not observe an inhibitory effect of cilostazol on recombinant human PDE5 expressed in mammalian cells (unpublished observation). If indeed cilostazol elevates cGMP in cells involved in atherosclerosis, either via NO release or PDE5 inhibition, this effect could potentially contribute to its therapeutic efficacy, as cGMP has very broad biological effects (Kemp-Harper and Schmidt 2009; Tsai and Kass 2009), with some of these effects, including vasodilation, antiplatelet and endothelium protection, being similar to those of cAMP. Furthermore, cross-talk between cAMP and cGMP signaling pathways may also be involved in modulating these cellular functions (Zaccolo and Movsesian 2007). Section 5 discusses the attempts to evaluate potential benefits of cGMP elevation for the treatment of IC.

4.1.4 Minimal Bleeding Risk with Cilostazol

Thrombosis and hemostasis share many similar molecular events. Thus, antiplatelet therapies are usually associated with increased risk of bleeding. Cilostazol seems to be an exception. Clinical studies have demonstrated no increase in bleeding risk in patients treated with cilostazol, and no added risk if co-administered with other antiplatelet agents, such as aspirin and clopidogrel (see review by Goto 2005). The lack of effect on bleeding is important as an increase in such events is associated with major ischemic complications in PAD patients (van Hattum et al. 2009). There is no obvious explanation for this unique property of cilostazol. Further studies are warranted to understand this unique feature of cilostazol so the knowledge can be applied in future drug development.

4.1.5 Class Effect of PDE3 Inhibitor on the Heart

The development of PDE3 inhibitors has been hindered by the observation that some inhibitors including milrinone worsen mortality in heart failure patients [more details in Movsesian and Kukreja (2011)]. Because of this, patients with heart failure with any severity were excluded in clinical trials for cilostazol, and its use has been contraindicated in patients with heart failure. All cause and cardiovascular mortality were compared in IC (excluding those with heart failure) patients taking cilostazol or placebo in a phase IV study (Hiatt et al. 2008). No indication of increased mortality was associated with the use of cilostazol in these patients. The finding that cilostazol elevates extracellular adenosine has been suggested to alleviate any potentially toxic effects arising from excessive cAMP elevation caused by PDE3 inhibition in the heart (Wang et al. 2001). This can be explained by the predominance of adenosine A_1 receptors, which inhibits AC via the stimulation of G_i , in cardiac myocytes (Kambayashi and Liu 2007).

4.1.6 Summary

Cilostazol has multiple activities; some are mediated through inhibition of PDE3, while others are caused by different mechanisms. The inhibitory effect of cilostazol on PDE3, together with its effects on signaling through adenosine, prostaglandins and NO on platelets, VSMCs, and endothelium (Fig. 5), likely contributes to its overall benefits in IC patients. Nevertheless, the exact underlying mechanisms are still unidentified. Direct measurement of blood flow after cilostazol treatment has not been attempted in IC patients. Using ABI as an indirect evaluation of blood supply to the leg, one study showed an improvement after several weeks of treatment with cilostazol (Mohler et al. 2001), while another study demonstrated no effect (Dawson et al. 1998). The increase in walking distance by cilostazol also required weeks of treatment (Beebe et al. 1999; Dawson et al. 1998), suggesting

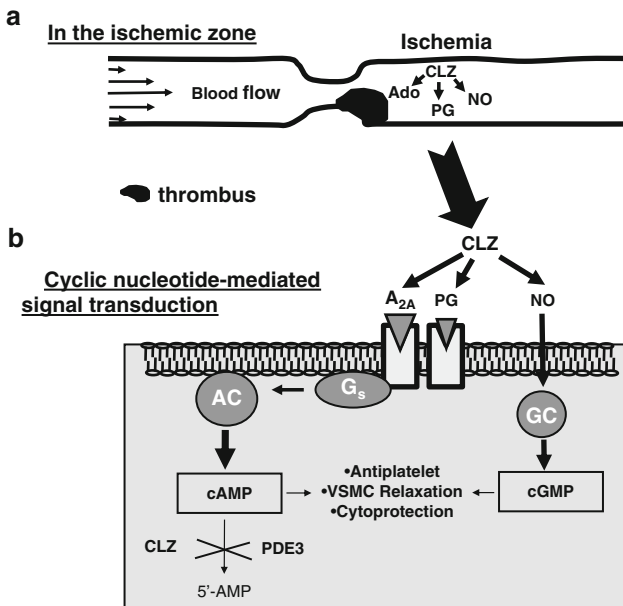


Fig. 5 Possible PDE-independent actions for cilostazol in the ischemic zone (a) and potential actions relating to PDE3 inhibition in the affected cells (including VSMCs, platelets, and endothelium) (b). A: Atherosclerosis results in the stenosis and narrowing of the artery. Thrombi can also form downstream of the lesion site. In the IC patients, ischemia can be induced during walking/exercise due to these pathological changes. Cilostazol can increase extracellular adenosine (Ado), prostaglandin (PG) E₁ and I₂, and nitric oxide (NO) concentration. B: By activating Ado A_{2A} and prostaglandin receptors, adenosine and prostaglandin(s) stimulate adenylyl cyclases (AC) to generate cAMP, which can be further elevated by the inhibition of PDE3. NO stimulates cellular soluble GC that generates cGMP. cAMP and cGMP both have anti-aggregatory effects on platelets, promote vasodilation and have cytoprotective properties. These effects likely work in concert to produce the clinical benefits of cilostazol in IC patients. CLZ cilostazol, VSMC vascular smooth muscle cells, G_s stimulatory G-protein

a progressive modulation of the disease state. Thus far, no studies have investigated the effect of cilostazol on muscle metabolism. While cilostazol can relieve symptoms associated with IC, it does not reverse or cure the disease and it has not been associated with any improvement in mortality in the IC population (Beebe et al. 1999; Hiatt et al. 2008).

4.2 *Pentoxifylline*

Pentoxifylline was the first drug specifically approved for increasing walking distance in IC patients and is a weak, nonselective PDE inhibitor (Table 1). Mega-analyses of clinical trials found a marginal but statistically significant improvement in pain-free and maximal walking distance (Girolami et al. 1999; Moher et al. 2000). Little is known about the mechanisms involved, but some studies have suggested that pentoxifylline and its metabolites improve the flow properties of blood by decreasing its viscosity (Ambrus et al. 1984; Berman et al. 1994; Ehrly 1978; Ferraresi et al. 1983; Ohshima and Sato 1981). This can theoretically increase blood flow to the affected microcirculation and enhance tissue oxygenation in patients with chronic PAD. However, in a more recent study, no such modification of blood rheologic properties was found in patients treated with pentoxifylline (Dawson et al. 2002). Regardless of the mechanism of action, inhibition of PDEs by pentoxifylline, which occurs with low potency and specificity, is unlikely to provide a significant contribution to pentoxifylline's effects.

4.3 *Other PDE3 Inhibitors Entering the IC Arena*

Encouraged by the success of cilostazol, companies especially in Japan are developing new and more potent PDE3 inhibitors for IC. Nissan Chemical and Taisho of Japan have evaluated NT-702 (NM-702, parogrelil, Fig. 4) for the treatment of IC. NT-702 inhibits PDE3 very potently. It also inhibits PDE5 and platelet TXA₂ synthase (Table 1). In a rat femoral artery ligation model, NT-702 increased walking distance (Ishiwata et al. 2007). In a Phase II study, treatment with NT-702 for 24 weeks was associated with improvement in walking distance compared with placebo (Brass et al. 2006). The improvement was very similar to that observed with the use of cilostazol. As with cilostazol, there was no significant bleeding risk associated with the use of NT-702 in this study (Brass et al. 2006).

Another Japanese company, Kowa Pharmaceuticals, has developed a PDE3 inhibitor K-134 (OPC-33509, Fig. 3) which is under Phase II trial for IC. This compound is a derivative of cilostazol and its inhibitory potency for PDE3 is similar to that of cilostazol (Koga et al. 1998; Sudo et al. 2000) (Table 1). It is unknown whether K-134 has any inhibitory effect on adenosine uptake, but it seems to possess additional PDE-unrelated activity as one study found that it inhibited the

binding of CD36 to type 1 collagen, whilst other PDE3 inhibitors do not (Ikenoya et al. 2007).

5 Targeting PDEs for Treatment of IC in Future Drug Development

Current pharmacotherapies for IC are limited and outcomes are far from optimal with only modest relief of symptoms. None can reverse the progression of the disease. In future drug development, PDE3 should still be considered a prime target, as inhibition has a plethora of benefits on platelets and the vasculature. However, the association of PDE3 inhibitors with unwanted cardiac effects and the assumed class effect in heart failure patients (worsening of mortality) makes PDE3 inhibition a potential safety liability. Recent advances in studies on intracellular compartmentalization of PDE isoforms in cardiac myocytes suggest that it is possible to generate cAMP levels in local microdomains (Fischmeister et al. 2006; Hambleton et al. 2005; Movsesian 2002). This characteristic may enable separation of the inotropic effect of PDE3 inhibitors from the arrhythmogenic and other detrimental effects on the heart. However, designing isoform-selective PDE inhibitors has proven difficult (Thompson et al. 2007) and identification of spatially selective inhibitors (i.e., only inhibiting PDE3 in one compartment of the cell) is likely to be even more challenging.

Synthetic analogues of prostacyclins such as beraprost and iloprost have been evaluated on the basis of their ability to activate Gs and increase intracellular cAMP in endothelium, platelets, and VSMCs. Some improvement in walking distance was observed in small clinical studies (Lievre et al. 2000; Muller-Buhl et al. 1987), but larger and two recent studies failed to confirm the effect on walking distance (Creager et al. 2008; Mohler et al. 2003b).

Like cAMP, cGMP has also been a target for the treatment of IC, via the direct activation of the soluble GC by the NO donor, L-arginine. However, the overall benefits are largely negative (Boger et al. 1998; Oka et al. 2005; Wilson et al. 2007). Ataciguat (HMR-1766), a NO-independent, soluble GC activator, developed by Sanofi-Aventis (Schindler et al. 2006), was shown, in preclinical studies, to protect coronary endothelium against ischemia/reperfusion (Kasseckert et al. 2009) and inhibit platelet activation (Schafer et al. 2006). A clinical trial evaluated its effect on patients with IC (ACCELA, clinicaltrials.gov). The trial results have not been published, but in February 2009 development for this indication was terminated without explanation (<https://www.thomson-pharma.com>). Another mechanism for increasing intracellular cGMP is via inhibition of PDE5 as this enzyme is abundant in VSMC and vascular endothelium, and has also been shown to be abundant in platelets. In tissues thus far studied, PDE5 has the highest expression in the VSMCs of the corpus cavernosum of the penis (Morelli et al. 2004), allowing inhibitors to be effective in the treatment of male erectile dysfunction. However, at this time, PDE5 inhibitors have not been evaluated in IC.

6 Combinatorial Therapies for the Future

PAD including IC is often caused by underlying arterial atherosclerosis, and is a complex disease resulting from multiple molecular abnormalities. Thus, targeting a single defect by highly specific drugs may be less effective than using more promiscuous or “dirty” drugs in treating such complex diseases (Frantz 2005). Cilostazol may be an example of this latter category of drug because it has multiple actions including inhibition of PDE3 (which targets platelets, VSMCs, and endothelium), as well as increasing adenosine, prostaglandin, and NO concentrations. Another advantage of multitargeted therapy is the possibility that potential undesired side effects may be reduced through the interaction of different signaling pathways. For example, in the case of cilostazol, increased adenosine accumulation may reduce the positive cardiac inotropic and chronotropic effects caused by PDE3 inhibition (Liu et al. 2001; Wang et al. 2001).

A multitargeted approach can also be achieved through treatment of completely different disease components. An on-going clinical trial is evaluating the effects of combining cilostazol with L-carnitine (Clinicaltrials.gov) on walking distance in patients with IC. Because cilostazol and L-carnitine may modify different aspects of the disease, a combined therapy may have added benefits. Several studies have investigated the effects of statins on walking distance and exercise performance in IC patients. These studies reported either an improvement in walking distance (Mohler et al. 2003a; Mondillo et al. 2003), or no effect (Bregar et al. 2009). Statins are unlikely to have a direct effect on platelet and vascular functions, but the improvement in lipid profile by statins may reduce the progression of atherosclerosis, thus alleviating the symptoms of IC. Since cilostazol and statins target different cellular mechanisms, it is possible that a combination therapy may provide added benefit. Indeed, two studies provide support for such a combination: one demonstrated that cilostazol and atorvastatin protect the ischemic heart in a synergistic fashion (Manickavasagam et al. 2007), and the other demonstrated that a combination of cilostazol and probucol, which lowers cholesterol by a different mechanism from statins, was more effective in preventing atherosclerotic lesion formation than the administration of each drug alone (Yoshikawa et al. 2008).

7 Summary

Intermittent claudication is most commonly caused by atherosclerotic arteries in the leg limiting the patient’s ability to walk and exercise and severely reducing quality of life. PDE3 is highly expressed in the cardiovascular system and inhibitors of this enzyme have a plethora of effects that are beneficial for treatment of IC, including the inhibition of platelet activation, relaxation of VSMCs, inhibition of VSMC proliferation, and the modulation of lipid profiles. One of these inhibitors, cilostazol, is approved for this indication as it significantly increases walking

distance in IC patients. Inhibition of adenosine uptake by cilostazol may also contribute to its efficacy and safety. While PDE3 inhibition is still being pursued for the treatment of IC, improved treatment in the future may result from pharmacological targeting of multiple pathways. Finally, an optimal pharmacological treatment should relieve both the symptoms of IC and reduce cardiovascular morbidity and mortality.

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References

- Ambrus JL, Ambrus CM, Taheri SA, Gastpar H, Reddington MM, Taheri P, Kahn EA, Schattman GL, Dean LS, Moore RH (1984) Red cell flexibility and platelet aggregation in patients with chronic obstructive vascular disease (COAD) and study of therapeutic approaches. *Angiology* 35:418–426
- Ashikaga T, Strada SJ, Thompson WJ (1997) Altered expression of cyclic nucleotide phosphodiesterase isozymes during culture of aortic endothelial cells. *Biochem Pharmacol* 54:1071–1079
- Askew CD, Green S, Walker PJ, Kerr GK, Green AA, Williams AD, Febbraio MA (2005) Skeletal muscle phenotype is associated with exercise tolerance in patients with peripheral arterial disease. *J Vasc Surg* 41:802–807
- Barker GA, Green S, Askew CD, Green AA, Walker PJ (2001) Effect of propionyl-L-carnitine on exercise performance in peripheral arterial disease. *Med Sci Sports Exerc* 33:1415–1422
- Barton M, Haudenschild CC (2001) Endothelium and atherogenesis: endothelial therapy revisited. *J Cardiovasc Pharmacol* 38(Suppl 2):S23–S25
- Beebe HG, Dawson DL, Cutler BS, Herd JA, Strandness DEJ, Bortey EB, Forbes WP (1999) A new pharmacological treatment for intermittent claudication: results of a randomized, multicenter trial. *Arch Intern Med* 159:2041–2050
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Berman W Jr, Berman N, Pathak D, Wood SC (1994) Effects of pentoxifylline (Trental) on blood flow, viscosity, and oxygen transport in young adults with inoperable cyanotic congenital heart disease. *Pediatr Cardiol* 15:66–70
- Boger RH, Bode-Boger SM, Thiele W, Creutzig A, Alexander K, Frolich JC (1998) Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. *J Am Coll Cardiol* 32:1336–1344
- Brass EP, Anthony R, Cobb FR, Koda I, Jiao J, Hiatt WR (2006) The novel phosphodiesterase inhibitor NM-702 improves claudication-limited exercise performance in patients with peripheral arterial disease. *J Am Coll Cardiol* 48:2539–2545
- Bregar U, Poredos P, Sabovic M, Jug B, Sebestjen M (2009) The influence of atorvastatin on walking performance in peripheral arterial disease. *Vasa* 38:155–159
- Cheung P, Yang G, Boden G (2003) Milrinone, a selective phosphodiesterase 3 inhibitor, stimulates lipolysis, endogenous glucose production, and insulin secretion. *Metabolism* 52:1496–1500
- Chiu PJ, Vemulapalli S, Chintala M, Kurowski S, Tetzloff GG, Brown AD, Sybertz EJ (1997) Inhibition of platelet adhesion and aggregation by E4021, a type V phosphodiesterase inhibitor, in guinea pigs. *Naunyn Schmiedebergs Arch Pharmacol* 355:463–469
- Choi JM, Shin HK, Kim KY, Lee JH, Hong KW (2002) Neuroprotective effect of cilostazol against focal cerebral ischemia via antiapoptotic action in rats. *J Pharmacol Exp Ther* 300:787–793

- Choi YH, Park S, Hockman S, Zmuda-Trzebiatowska E, Svennelid F, Haluzik M, Gavrilova O, Ahmad F, Pepin L, Napolitano M, Taira M, Sundler F, Stenson HL, Degerman E, Manganiello VC (2006) Alterations in regulation of energy homeostasis in cyclic nucleotide phosphodiesterase 3B-null mice. *J Clin Invest* 116:3240–3251
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- Creager MA, Pande RL, Hiatt WR (2008) A randomized trial of iloprost in patients with intermittent claudication. *Vasc Med* 13:5–13
- Dal LA, De MD, Flore R, Gaetani E, Gasbarrini A, Gerardino L, Pola R, Santoliquido A, Serricchio M, Tondi P, Nolfo G (1999) Effects of propionyl-L-carnitine on peripheral arterial obliterative disease of the lower limbs: a double-blind clinical trial. *Drugs Exp Clin Res* 25:29–36
- Dawson DL, Cutler BS, Meissner MH, Strandness DEJ (1998) Cilostazol has beneficial effects in treatment of intermittent claudication: results from a multicenter, randomized, prospective, double-blind trial. *Circulation* 98:678–686
- Dawson DL, Cutler BS, Hiatt WR, Hobson RW, Martin JD, Bortey EB, Forbes WP, Strandness DE (2000) A comparison of cilostazol and pentoxifylline for treating intermittent claudication. *Am J Med* 109:523–530
- Dawson DL, Zheng Q, Worthy SA, Charles B, Bradley DV Jr (2002) Failure of pentoxifylline or cilostazol to improve blood and plasma viscosity, fibrinogen, and erythrocyte deformability in claudication. *Angiology* 53:509–520
- de la Cruz JP, Romero MM, Sanchez P, de la Sanchez CF (1993) Antiplatelet effect of pentoxifylline in human whole blood. *Gen Pharmacol* 24:605–609
- Dimmeler S, Haendeler J, Galle J, Zeiher AM (1997) Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases: a mechanistic clue to the 'response to injury' hypothesis. *Circulation* 95:1760–1763
- Douglas JS Jr, Holmes DR Jr, Kereiakes DJ, Grines CL, Block E, Ghazzal ZM, Morris DC, Liberman H, Parker K, Jurkovitz C, Murrah N, Foster J, Hyde P, Mancini GB, Weintraub WS (2005) Coronary stent restenosis in patients treated with cilostazol. *Circulation* 112:2826–2832
- Dunkel B, Rickards KJ, Page CP, Cunningham FM (2007) Phosphodiesterase isoenzymes in equine platelets and their influence on platelet adhesion. *Am J Vet Res* 68:1354–1360
- Dunkern TR, Hatzelmann A (2005) The effect of Sildenafil on human platelet secretory function is controlled by a complex interplay between phosphodiesterases 2, 3 and 5. *Cell Signal* 17:331–339
- Ehrly AM (1978) The effect of pentoxifylline on the flow properties of human blood. *Curr Med Res Opin* 5:608–613
- Elam MB, Heckman J, Crouse JR, Hunninghake DB, Herd JA, Davidson M, Gordon IL, Bortey EB, Forbes WP (1998) Effect of the novel antiplatelet agent cilostazol on plasma lipoproteins in patients with intermittent claudication. *Arterioscler Thromb Vasc Biol* 18:1942–1947
- Ferraresi I, Bozzini F, Torta D, Frigerio R, Bernasconi C, Agostoni A (1983) In vitro effects of pentoxifylline on hemoglobin affinity for oxygen and electrolytic equilibrium of human blood. *Ric Clin Lab* 13:459–465
- Fischmeister R, Castro LR, bi-Gerges A, Rochais F, Jurevicius J, Leroy J, Vandecasteele G (2006) Compartmentation of cyclic nucleotide signaling in the heart: the role of cyclic nucleotide phosphodiesterases. *Circ Res* 99:816–828
- Fong M, Yoshitake M, Kambayashi J, Liu Y (2010) Cilostazol increases tissue blood flow in contracting rabbit gastrocnemius muscle. *Circ J* 74:181–187
- Frantz S (2005) Drug discovery: playing dirty. *Nature* 437:942–943
- Fukasawa M, Nishida H, Sato T, Miyazaki M, Nakaya H (2008) 6-[4-(1-Cyclohexyl-1H-tetrazol-5-yl)butoxy]-3, 4-dihydro-2-(1H)quinolinone (cilostazol), a phosphodiesterase type 3 inhibitor, reduces infarct size via activation of mitochondrial Ca²⁺-activated K⁺ channels in rabbit hearts. *J Pharmacol Exp Ther* 326:100–104

- Galle J, Lehmann-Bodem C, Hubner U, Heinloth A, Wanner C (2000) CyA and OxLDL cause endothelial dysfunction in isolated arteries through endothelin-mediated stimulation of O₂- formation. *Nephrol Dial Transplant* 15:339–346
- Girolami B, Bernardi E, Prins MH, Ten Cate JW, Hettiarachchi R, Prandoni P, Girolami A, Buller HR (1999) Treatment of intermittent claudication with physical training, smoking cessation, pentoxifylline, or naftonyl: a meta-analysis. *Arch Intern Med* 159:337–345
- Goto S (2005) Cilostazol: potential mechanism of action for antithrombotic effects accompanied by a low rate of bleeding. *Atheroscler Suppl* 6:3–11
- Greenhaff PL, Campbell-O'Sullivan SP, Constantin-Teodosiu D, Poucher SM, Roberts PA, Timmons JA (2004) Metabolic inertia in contracting skeletal muscle: a novel approach for pharmacological intervention in peripheral vascular disease. *Br J Clin Pharmacol* 57: 237–243
- Gudmundsdottir IJ, McRobbie SJ, Robinson SD, Newby DE, Megson IL (2005) Sildenafil potentiates nitric oxide mediated inhibition of human platelet aggregation. *Biochem Biophys Res Commun* 337:382–385
- Hambleton R, Krall J, Tikishvili E, Honegger M, Ahmad F, Manganiello VC, Movsesian MA (2005) Isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP hydrolytic activity in subcellular fractions of human myocardium. *J Biol Chem* 280:39168–39174
- Hankey GJ, Norman PE, Eikelboom JW (2006) Medical treatment of peripheral arterial disease. *JAMA* 295:547–553
- Hashimoto A, Miyakoda G, Hirose Y, Mori T (2006) Activation of endothelial nitric oxide synthase by cilostazol via a cAMP/protein kinase A- and phosphatidylinositol 3-kinase/Akt-dependent mechanism. *Atherosclerosis* 189:350–357
- Hiatt WR (2001) Medical treatment of peripheral arterial disease and claudication. *N Engl J Med* 344:1608–1621
- Hiatt WR (2004) Carnitine and peripheral arterial disease. *Ann NY Acad Sci* 1033:92–98
- Hiatt WR, Regensteiner JG, Creager MA, Hirsch AT, Cooke JP, Olin JW, Gorbunov GN, Isner J, Lukjanov YV, Tsitsiashvili MS, Zabelskaya TF, Amato A (2001) Propionyl-L-carnitine improves exercise performance and functional status in patients with claudication. *Am J Med* 110:616–622
- Hiatt WR, Money SR, Brass EP (2008) Long-term safety of cilostazol in patients with peripheral artery disease: the CASTLE study (Cilostazol: A Study in Long-term Effects). *J Vasc Surg* 47:330–336
- Hirsch AT, Haskal ZJ, Hertzner NR, Bakal CW, Creager MA, Halperin JL, Hiratzka LF, Murphy WRC, Olin JW, Puschett JB, Rosenfield KA, Sacks D, Stanley JC, Taylor LM Jr, White CJ, White J, White RA (2006) ACC/AHA 2005 practice guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic). *Circulation* 113:e463–e465
- Honda F, Imai H, Ishikawa M, Kubota C, Shimizu T, Fukunaga M, Saito N (2006) Cilostazol attenuates gray and white matter damage in a rodent model of focal cerebral ischemia. *Stroke* 37:223–228
- Igawa T, Tani T, Chijiwa T, Shiragiku T, Shimidzu S, Kawamura K, Kato S, Unemi F, Kimura Y (1990) Potentiation of anti-platelet aggregating activity of cilostazol with vascular endothelial cells. *Thromb Res* 57:617–623
- Ikeda U, Ikeda M, Kano S, Kanbe T, Shimada K (1996) Effect of cilostazol, a cAMP phosphodiesterase inhibitor, on nitric oxide production by vascular smooth muscle cells. *Eur J Pharmacol* 314:197–202
- Ikenoya M, Doi T, Miura T, Sawanobori K, Nishio M, Hidaka H (2007) Investigation of binding proteins for anti-platelet agent K-134 by Drug-Western method. *Biochem Biophys Res Commun* 353:1111–1114
- Ikewaki K, Mochizuki K, Iwasaki M, Nishide R, Mochizuki S, Tada N (2002) Cilostazol, a potent phosphodiesterase type III inhibitor, selectively increases antiatherogenic high-density

- lipoprotein subclass LpA-I and improves postprandial lipemia in patients with type 2 diabetes mellitus. *Metabolism* 51:1348–1354
- Inada H, Shindo H, Tawata M, Onaya T (1999) Cilostazol, a cyclic AMP phosphodiesterase inhibitor, stimulates nitric oxide production and sodium potassium adenosine triphosphatase activity in SH-SY5Y human neuroblastoma cells. *Life Sci* 65:1413–1422
- Indolfi C, Di LE, Rapacciuolo A, Stingone AM, Stabile E, Leccia A, Torella D, Caputo R, Ciardiello F, Tortora G, Chiariello M (2000) 8-chloro-cAMP inhibits smooth muscle cell proliferation in vitro and neointima formation induced by balloon injury in vivo. *J Am Coll Cardiol* 36:288–293
- Ishikawa M, Yamada Y, Hirose C, Tujino D, Hoshi K, Saitou N (1997) The effects of cilostazol on serum lipid metabolism and ASO in NIDDM with hypertriglyceridemia. *Ther Res* 18:198–204
- Ishiwata N, Noguchi K, Kawanishi M, Asakura Y, Hori M, Mitani A, Ito Y, Takahashi K, Nishiyama H, Shudo N, Takahashi S, Takahashi K, Tsuruzoe N, Nakaie S (2007) NT-702 (paregrelil hydrochloride, NM-702), a novel and potent phosphodiesterase inhibitor, improves reduced walking distance and lowered hindlimb plantar surface temperature in a rat experimental intermittent claudication model. *Life Sci* 81:970–978
- Ito H, Hashimoto A, Matsumoto Y, Yao H, Miyakoda G (2010) Cilostazol, a phosphodiesterase inhibitor, attenuates photothrombotic focal ischemic brain injury in hypertensive rats. *J Cereb Blood Flow Metab* 30:343–351
- Iwama D, Miyamoto K, Miyahara S, Tamura H, Tsujikawa A, Yamashiro K, Kiryu J, Yoshimura N (2007) Neuroprotective effect of cilostazol against retinal ischemic damage via inhibition of leukocyte-endothelial cell interactions. *J Thromb Haemost* 5:818–825
- Jackson SP, Nesbitt WS, Westein E (2009) Dynamics of platelet thrombus formation. *J Thromb Haemost* 7(Suppl 1):17–20
- Kabayashi SY, Liu Y (2007) Bench to bedside: multiple actions of PDE3 inhibitor cilostazol. In: Beavo JA, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, FL, pp 627–648
- Kabayashi J, Liu Y, Sun B, Shakur Y, Yoshitake M, Czerwiec F (2003) Cilostazol as a unique antithrombotic agent. *Curr Pharm Des* 9:2289–2308
- Kassekert SA, Schafer C, Kluger A, Gligorievski D, Tillmann J, Schluter KD, Noll T, Sauer H, Piper HM, Abdallah Y (2009) Stimulation of cGMP signalling protects coronary endothelium against reperfusion-induced intercellular gap formation. *Cardiovasc Res* 83:381–387
- Kemp-Harper B, Schmidt HH (2009) cGMP in the vasculature. *Handb Exp Pharmacol* 191:447–467
- Keravis SAP, Favot L, Lugnier C (2007) Role of PDEs in vascular health and disease: endothelial PDEs and angiogenesis. In: Beavo JA, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, FL, pp 417–439
- Kim KY, Shin HK, Choi JM, Hong KW (2002) Inhibition of lipopolysaccharide-induced apoptosis by cilostazol in human umbilical vein endothelial cells. *J Pharmacol Exp Ther* 300:709–715
- Kimura Y, Tani T, Kanbe T, Watanabe K (1985) Effect of cilostazol on platelet aggregation and experimental thrombosis. *Arzneimittelforschung* 35:1144–1149
- Koga Y, Kihara Y, Okada M, Inoue Y, Tochizawa S, Toga K, Tachibana K, Kimura Y, Nishi T, Hidaka H (1998) 2(1H)-quinolinone derivatives as novel anti-arteriosclerotic agents showing anti-thrombotic and anti-hyperplastic activities. *Bioorg Med Chem Lett* 8:1471–1476
- Lee JH, Kim KY, Lee YK, Park SY, Kim CD, Lee WS, Rhim BY, Hong KW (2004) Cilostazol prevents focal cerebral ischemic injury by enhancing casein kinase 2 phosphorylation and suppression of phosphatase and tensin homolog deleted from chromosome 10 phosphorylation in rats. *J Pharmacol Exp Ther* 308:896–903
- Lievre M, Morand S, Besse B, Fiessinger JN, Boissel JP (2000) Oral Beraprost sodium, a prostaglandin I₂ analogue, for intermittent claudication: a double-blind, randomized, multicenter controlled trial. *Beraprost et Claudication Intermittente (BERCI) Research Group. Circulation* 102:426–431

- Liu Y, Fong M, Cone J, Wang S, Yoshitake M, Kambayashi J (2000) Inhibition of adenosine uptake and augmentation of ischemia-induced increase of interstitial adenosine by cilostazol, an agent to treat intermittent claudication. *J Cardiovasc Pharmacol* 36:351–360
- Liu Y, Shakur Y, Yoshitake M, Kambayashi JJ (2001) Cilostazol (pletal): a dual inhibitor of cyclic nucleotide phosphodiesterase type 3 and adenosine uptake. *Cardiovasc Drug Rev* 19:369–386
- Loscalzo J (2001) Nitric oxide insufficiency, platelet activation, and arterial thrombosis. *Circ Res* 88:756–762
- Madamanchi NR, Vendrov A, Runge MS (2005) Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol* 25:29–38
- Manickavasagam S, Ye Y, Lin Y, Perez-Polo RJ, Huang MH, Lui CY, Hughes MG, McAdoo DJ, Uretsky BF, Birnbaum Y (2007) The cardioprotective effect of a statin and cilostazol combination: relationship to Akt and endothelial nitric oxide synthase activation. *Cardiovasc Drugs Ther* 21:321–330
- Maurice DH (2005) Cyclic nucleotide phosphodiesterase-mediated integration of cGMP and cAMP signaling in cells of the cardiovascular system. *Front Biosci* 10:1221–1228
- Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR, Elbatarny HS, Jimmo SL (2003) Cyclic nucleotide phosphodiesterase activity, expression, and targeting in cells of the cardiovascular system. *Mol Pharmacol* 64:533–546
- Mishima Y, Kuyama A, Ando M, Kibata M, Ishioka T (2000) Effects of cilostazol on serum lipoprotein concentrations and particle size of low-density lipoproteins in patients with dyslipidemic NIDDM. *J Jpn Atheroscler Soc* 27:17–22
- Mizutani M, Okuda Y, Yamashita K (1996) Effect of cilostazol on the production of platelet-derived growth factor in cultured human vascular endothelial cells. *Biochem Mol Med* 57:156–158
- Moher D, Pham B, Ausejo M, Saenz A, Hood S, Barber GG (2000) Pharmacological management of intermittent claudication: a meta-analysis of randomised trials. *Drugs* 59:1057–1070
- Mohler ER III, Beebe HG, Salles-Cuhna S, Zimet R, Zhang P, Heckman J, Forbes WP (2001) Effects of cilostazol on resting ankle pressures and exercise-induced ischemia in patients with intermittent claudication. *Vasc Med* 6:151–156
- Mohler ER III, Hiatt WR, Creager MA (2003a) Cholesterol reduction with atorvastatin improves walking distance in patients with peripheral arterial disease. *Circulation* 108:1481–1486
- Mohler ER III, Hiatt WR, Olin JW, Wade M, Jeffs R, Hirsch AT (2003b) Treatment of intermittent claudication with beraprost sodium, an orally active prostaglandin I₂ analogue: a double-blinded, randomized, controlled trial. *J Am Coll Cardiol* 41:1679–1686
- Mondillo S, Ballo P, Barbati R, Guerrini F, Ammataro T, Agricola E, Pastore M, Borrello F, Belcastro M, Picchi A, Nami R (2003) Effects of simvastatin on walking performance and symptoms of intermittent claudication in hypercholesterolemic patients with peripheral vascular disease. *Am J Med* 114:359–364
- Morelli A, Filippi S, Mancina R, Luconi M, Vignozzi L, Marini M, Orlando C, Vannelli GB, Aversa A, Natali A, Forti G, Giorgi M, Jannini EA, Ledda F, Maggi M (2004) Androgens regulate phosphodiesterase type 5 expression and functional activity in corpora cavernosa. *Endocrinology* 145:2253–2263
- Mori D, Ishii H, Kojima C, Nitta N, Nakajima K, Yoshida M (2007) Cilostazol inhibits monocytic cell adhesion to vascular endothelium via upregulation of cAMP. *J Atheroscler Thromb* 14:213–218
- Movsesian MA (2002) PDE3 cyclic nucleotide phosphodiesterases and the compartmentation of cyclic nucleotide-mediated signalling in cardiac myocytes. *Basic Res Cardiol* 97(Suppl 1): 183–190
- Movsesian MA, Kukreja RC (2011) Phosphodiesterase inhibition in heart failure. In: Francis SH, Conti M, Houslay MD (eds) *Phosphodiesterases as drug targets*. Springer, Heidelberg
- Muller-Buhl U, Diehm C, Kraus T, Zimmermann R, Morl H, Eckstein HH (1987) Clinical effects of intravenous iloprost in patients with intermittent claudication. *Eur J Clin Pharmacol* 33:127–131

- Nakamura T, Houchi H, Minami A, Sakamoto S, Tsuchiya K, Niwa Y, Minakuchi K, Nakaya Y (2001) Endothelium-dependent relaxation by cilostazol, a phosphodiesterase III inhibitor, on rat thoracic aorta. *Life Sci* 69:1709–1715
- Nakamura N, Hamazaki T, Johkaji H, Minami S, Yamazaki K, Satoh A, Sawazaki S, Urakaze M, Kobayashi M, Osawa H, Yamabe H, Okomura K (2003) Effects of cilostazol on serum lipid concentrations and plasma fatty acid composition in type 2 diabetic patients with peripheral vascular disease. *Clin Exp Med* 2:180–184
- Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, Carberry J, Fouras A, Jackson SP (2009) A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nat Med* 15:665–673
- Netherton SJ, Maurice DH (2005) Vascular endothelial cell cyclic nucleotide phosphodiesterases and regulated cell migration: implications in angiogenesis. *Mol Pharmacol* 67:263–272
- Nieswandt B, Watson SP (2003) Platelet-collagen interaction: is GPVI the central receptor? *Blood* 102:449–461
- Nishi T, Tabusa F, Tanaka T, Shimizu T, Nakagawa K (1985) Studies on 2-oxoquinoline derivatives as blood platelet aggregation inhibitors, IV. Synthesis and biological activity of the metabolites of 6-[4-(1-cyclohexyl-1H-5-tetrazolyl)butoxy]-2-oxo-1, 2, 3, 4-tetrahydroquinoline (OPC-13013). *Chem Pharm Bull (Tokyo)* 33:1140–1147
- Ohshima N, Sato M (1981) Effect of pentoxifylline on microvascular blood flow velocity. *Angiology* 32:752–763
- Oka RK, Szuba A, Giacomini JC, Cooke JP (2005) A pilot study of L-arginine supplementation on functional capacity in peripheral arterial disease. *Vasc Med* 10:265–274
- Omi H, Okayama N, Shimizu M, Fukutomi T, Nakamura A, Imaeda K, Okouchi M, Itoh M (2004) Cilostazol inhibits high glucose-mediated endothelial-neutrophil adhesion by decreasing adhesion molecule expression via NO production. *Microvasc Res* 68:119–125
- Osinski MT, Schror K (2000) Inhibition of platelet-derived growth factor-induced mitogenesis by phosphodiesterase 3 inhibitors: role of protein kinase A in vascular smooth muscle cell mitogenesis. *Biochem Pharmacol* 60:381–387
- Page CP, Spina D (2011) PDE inhibitors in the treatment of inflammatory diseases. In: Francis SH, Conti M, Houslay MD (eds) *Phosphodiesterases as drug targets*. Springer, Heidelberg
- Sanz MJ, Cortijo J, Taha MA, Cerda-Nicolas M, Schatton E, Burgbacher B, Klar J, Tenor H, Schudt C, Issekutz AC, Hatzelmann A, Morcillo EJ (2007) Roflumilast inhibits leukocyte-endothelial cell interactions, expression of adhesion molecules and microvascular permeability. *Br J Pharmacol* 152:481–492
- Schafer A, Flierl U, Kobsar A, Eigenthaler M, Ertl G, Bauersachs J (2006) Soluble guanylyl cyclase activation with HMR1766 attenuates platelet activation in diabetic rats. *Arterioscler Thromb Vasc Biol* 26:2813–2818
- Schindler U, Strobel H, Schonafinger K, Linz W, Lohn M, Martorana PA, Rutten H, Schindler PW, Busch AE, Sohn M, Topfer A, Pistorius A, Jannek C, Mulsch A (2006) Biochemistry and pharmacology of novel anthranilic acid derivatives activating heme-oxidized soluble guanylyl cyclase. *Mol Pharmacol* 69:1260–1268
- Schmidt U, Han RO, DiSalvo TG, Guerrero JL, Gold HK, Zapol WM, Bloch KD, Semigran MJ (2001) Cessation of platelet-mediated cyclic canine coronary occlusion after thrombolysis by combining nitric oxide inhalation with phosphodiesterase-5 inhibition. *J Am Coll Cardiol* 37:1981–1988
- Schwarz UR, Walter U, Eigenthaler M (2001) Taming platelets with cyclic nucleotides. *Biochem Pharmacol* 62:1153–1161
- Snyder PB (1999) The adipocyte of cGMP-inhibited cyclic nucleotide phosphodiesterase (PDE3B) as a target for lipolytic and thermogenic agents for the treatment of obesity. *Emerg Ther Targets* 3:587–599
- Snyder PB, Esselstyn JM, Loughney K, Wolda SL, Florio VA (2005) The role of cyclic nucleotide phosphodiesterases in the regulation of adipocyte lipolysis. *J Lipid Res* 46:494–503

- Stewart KJ, Hiatt WR, Regensteiner JG, Hirsch AT (2002) Exercise training for claudication. *N Engl J Med* 347:1941–1951
- Sudo T, Tachibana K, Toga K, Tochizawa S, Inoue Y, Kimura Y, Hidaka H (2000) Potent effects of novel anti-platelet aggregatory cilostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity. *Biochem Pharmacol* 59:347–356
- Sun B, Le SN, Lin S, Fong M, Guertin M, Liu Y, Tandon NN, Yoshitake M, Kambayashi J (2002) New mechanism of action for cilostazol: interplay between adenosine and cilostazol in inhibiting platelet activation. *J Cardiovasc Pharmacol* 40:577–585
- Sun B, Li H, Shakur Y, Hensley J, Hockman S, Kambayashi J, Manganiello VC, Liu Y (2007) Role of phosphodiesterase type 3A and 3B in regulating platelet and cardiac function using subtype-selective knockout mice. *Cell Signal* 19:1765–1771
- Takayoshi T, Shinichi O, Ryuza A, Ryuichi S, Norihiro S, Hisamichi S, Akira F (2001) Effect of cilostazol on lipid, uric acid and glucose metabolism in patients with impaired glucose tolerance or type 2 diabetes mellitus: a double-blind, placebo-controlled study. *Clin Drug Investig* 21:325–335
- Tamai T, Shimada A, Maeda H, Takahashi S, Oida K, Nakai T, Miyabo S (1992) Reduction of low density lipoprotein by cilostazol in the non-insulin dependent diabetic patient. *Jpn Pharmacol Ther* 20:241–248
- Thompson WJ, Ashikaga T, Kelly JJ, Liu L, Zhu B, Vemavarapu L, Strada SJ (2002) Regulation of cyclic AMP in rat pulmonary microvascular endothelial cells by rolipram-sensitive cyclic AMP phosphodiesterase (PDE4). *Biochem Pharmacol* 63:797–807
- Thompson PE, Manganiello V, Degerman E (2007) Re-discovering PDE3 inhibitors—new opportunities for a long neglected target. *Curr Top Med Chem* 7:421–436
- Torii H, Kubota H, Ishihara H, Suzuki M (2007) Cilostazol inhibits the redistribution of the actin cytoskeleton and junctional proteins on the blood-brain barrier under hypoxia/reoxygenation. *Pharmacol Res* 55:104–110
- Traupe T, Ortmann J, Munter K, Barton M (2003) Endothelial therapy of atherosclerosis and its risk factors. *Curr Vasc Pharmacol* 1:111–121
- Tsai EJ, Kass DA (2009) Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics. *Pharmacol Ther* 122:216–238
- van Hattum ES, Algra A, Lawson JA, Eikelboom BC, Moll FL, Tangelder MJD (2009) Bleeding increases the risk of ischemic events in patients with peripheral arterial disease. *Circulation* 120:1569–1576
- Wakida K, Morimoto N, Shimazawa M, Hozumi I, Nagase H, Inuzuka T, Hara H (2006) Cilostazol reduces ischemic brain damage partly by inducing metallothionein-1 and -2. *Brain Res* 1116:187–193
- Wang S, Cone J, Fong M, Yoshitake M, Kambayashi J, Liu Y (2001) Interplay between inhibition of adenosine uptake and phosphodiesterase type 3 on cardiac function by cilostazol, an agent to treat intermittent claudication. *J Cardiovasc Pharmacol* 38:775–783
- Wang YX, Fitch R, Li W, Werner M, Halks-Miller M, Lillis B, Vergona R, Post J, Sullivan ME, Verhallen PF (2002) Reduction of cardiac functional reserve and elevation of aortic stiffness in hyperlipidemic Yucatan minipigs with systemic and coronary atherosclerosis. *Vascul Pharmacol* 39:69–76
- Wang T, Elam MB, Forbes WP, Zhong J, Nakajima K (2003) Reduction of remnant lipoprotein cholesterol concentrations by cilostazol in patients with intermittent claudication. *Atherosclerosis* 171:337–342
- Watanabe N, Ishikawa Y, Kitagawa Y, Ozaki M, Takeshita S, Namura H, Kurimoto Y, Yamada S, Yokoyama M (1996) Effects of cilostazol on the lipid metabolism in patients with hypertriglyceridemia. *Jpn Pharmacol Ther* 24:127–132
- Wilson AM, Harada R, Nair N, Balasubramanian N, Cooke JP (2007) L-arginine supplementation in peripheral arterial disease: no benefit and possible harm. *Circulation* 116:188–195
- Wilson LS, Elbatarny HS, Crawley SW, Bennett BM, Maurice DH (2008) Compartmentation and compartment-specific regulation of PDE5 by protein kinase G allows selective cGMP-mediated regulation of platelet functions. *Proc Natl Acad Sci USA* 105:13650–13655

- Yang Y, Luo J, Kazumura K, Takeuchi K, Inui N, Hayashi H, Ohashi K, Watanabe H (2006) Cilostazol suppresses adhesion of human neutrophils to HUVECs stimulated by FMLP and its mechanisms. *Life Sci* 79:629–636
- Yoshikawa T, Mitani K, Kotosai K, Nozako M, Miyakoda G, Yabuuchi Y (2008) Antiatherogenic effects of cilostazol and probucol alone, and in combination in low density lipoprotein receptor-deficient mice fed with a high fat diet. *Horm Metab Res* 40:473–478
- Yuzawa I, Yamada M, Fujii K (2008) An oral administration of cilostazol before focal ischemia reduces the infarct volume with delayed cerebral blood flow increase in rats. *J Stroke Cerebrovasc Dis* 17:281–286
- Zaccolo M, Movsesian MA (2007) cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res* 100:1569–1578
- Zhang W, Colman RW (2007) Thrombin regulates intracellular cyclic AMP concentration in human platelets through phosphorylation/activation of phosphodiesterase 3A. *Blood* 110:1475–1482
- Zhao H, Guan Q, Smith CJ, Quilley J (2008) Increased phosphodiesterase 3A/4B expression after angioplasty and the effect on VASP phosphorylation. *Eur J Pharmacol* 590:29–35
- Zhu B, Strada S, Stevens T (2005) Cyclic GMP-specific phosphodiesterase 5 regulates growth and apoptosis in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 289:L196–L206

Phosphodiesterase Inhibition in Heart Failure

Matthew A. Movsesian and Rakesh C. Kukreja

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Abstract Compounds that inhibit the catalytic activity of cyclic nucleotide phosphodiesterases are used as therapeutic agents to increase intracellular cAMP and/or cGMP content in cells or tissues of interest. In patients with heart failure, inhibitors of enzymes in the PDE3 family of cyclic nucleotide phosphodiesterases are used to raise intracellular cAMP content in cardiac muscle, with inotropic actions. These drugs are effective in acute applications, but their long-term use has been complicated by an increase in cardiovascular mortality in clinical trials. Inhibitors of enzymes in the PDE5 family have been used to raise cGMP content in cardiac muscle in animal models of pressure overload, chronic β -adrenergic receptor stimulation, ischemic injury, and doxorubicin toxicity, and have been shown to have antihypertrophic and cardioprotective actions. Recent experimental results raise some question as to the likely applicability of these findings to humans, in whose hearts PDE5 is present at much lower levels than those seen in animal models, and raise the possibility of PDE1, a dual-specificity phosphodiesterase

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present at high levels in human myocardium, as an alternative target for inotropic and cardioprotective actions.

Keywords cAMP · Cardiomyopathy · Cardioprotection · cGMP · Inotropy · PDE1 · PDE3 · PDE5 · PDE4

1 PDE3 Cyclic Nucleotide Phosphodiesterases in Human Myocardium

PDE3 isoforms constitute the majority of the membrane-associated cAMP-hydrolytic activity and a large fraction of the cytosolic cAMP-hydrolytic activity in human myocardium (Hambleton et al. 2005; Vandeput et al. 2007). These enzymes are dual-specificity enzymes, hydrolyzing both cAMP and cGMP with high affinities ($K_m \sim 0.1 \mu\text{M}$). Their catalytic rates for cAMP are much higher than their catalytic rates for cGMP; for this reason, they have generally been thought of primarily as cGMP-inhibited cAMP phosphodiesterases. Inhibition of the cAMP-hydrolytic activity of PDE3 by cGMP has been shown to be involved in its regulation of delayed-rectifier K^+ currents and L-type Ca^{2+} currents in cardiac myocytes (Shimizu et al. 2002; Frace et al. 1993; Vandecasteele et al. 2001), and there is evidence that cGMP may have inotropic effects in hypertrophic right ventricular myocardium that result from PDE3 inhibition (Nagendran et al. 2007). It should be noted, however, that the cGMP-hydrolytic activity of PDE3 comprises a significant fraction of the cGMP-hydrolytic activity recovered in soluble (cytosolic) and particulate (membrane-derived) fractions of human myocardium (Vandeput et al. 2007, 2009) (Fig. 1), and the possible role of these enzymes as cGMP phosphodiesterases has, to our knowledge, not been explored.

Within the PDE3 family, two genes – PDE3A and PDE3B – have been identified. Most of the PDE3 activity in cardiac myocytes appears to consist of three isoforms – PDE3A1, PDE3A2, and PDE3A3 – that appear to be generated from the *PDE3A* gene through transcription and translation from alternative start sites (Fig. 2) (Wechsler et al. 2002; Choi et al. 2001). As a result, the amino acid sequences of these isoforms are identical except for differences in the lengths of N-terminal sequence that are included. PDE3A1, the longest isoform, has thus far been found only in cardiac myocytes and oocytes; in the former, this isoform is recovered exclusively in membrane-derived particulate fractions (Han et al. 2006). PDE3A1 contains two membrane-localizing domains: “NHR1”, which consists of hydrophobic loops that insert into intracellular membranes, and “NHR2”, which appears to be important in protein–protein interactions (Kenan et al. 2000; Shakur et al. 2000). PDE3A1 also contains three sites – P1 (S293/S294), P2 (S312), and P3 (S428) – that are phosphorylated, with overlapping specificities, by PKA, PKB, and PKC. Two shorter isoforms, PDE3A2 and PDE3A3, are recovered in cytosolic as well as membrane-derived and fractions. PDE3A2 lacks NHR1 and the upstream phosphorylation site, while PDE3A3 lacks NHR1, NHR2, and the three phosphorylation sites. The three isoforms all contain the C-terminal catalytic region (CCR),

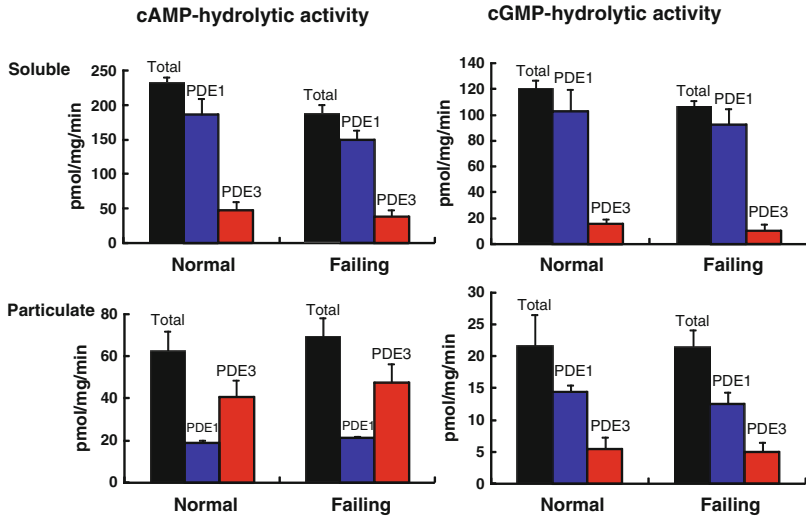


Fig. 1 cAMP- and cGMP-hydrolytic activity in soluble and particulate fractions of normal and failing human left ventricular myocardium. Assays were performed at 0.1 μ M cAMP

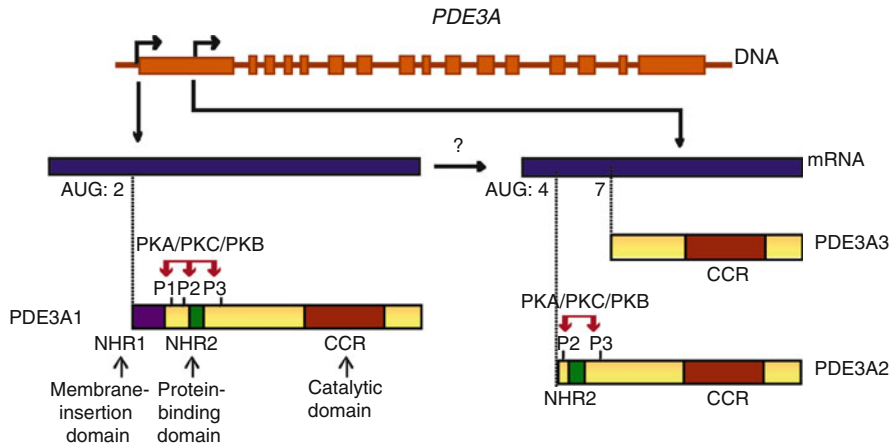


Fig. 2 PDE3A isoforms in human myocardium. Two messages appear to be generated by transcription from alternate sites in the first exon of *PDE3A*. The longer message appears to be translated from its second AUG to yield PDE3A1; the shorter message appears to be translated from downstream AUGs to yield PDE3A2 and PDE3A3

and are identical with respect to catalytic activity and inhibitor sensitivity (Hambleton et al. 2005).

There are conflicting reports regarding the level of PDE3 activity in normal and failing human myocardium. In one study, the expression of PDE3 was reported to be decreased in patients with dilated cardiomyopathy (Ding et al. 2005a).

Others have found that levels of PDE3 activity are more or less comparable in normal and failing human myocardium, or at least that the differences are not statistically significant (Vandeput et al. 2009; Movsesian et al. 1991). The reasons for the different results among these studies are not known.

2 PDE3 Inhibitors in the Treatment of Heart Failure

PDE3 inhibitors are used in the treatment of heart failure resulting from dilated cardiomyopathy, a disease of several etiologies that is characterized by chamber enlargement and a reduction in myocardial contractility related to a reduction in β -adrenergic receptor-stimulated cAMP generation (Bristow et al. 1986). By inhibiting cAMP hydrolysis in cardiac muscle, PDE3 inhibitors “overcome” the reduction in intracellular cAMP content and increase myocardial contractility (Baim et al. 1983; Sinoway et al. 1983; Uretsky et al. 1983; Jaski et al. 1985). PDE3 inhibition also raises cAMP content in vascular smooth muscle, where the consequence is relaxation of smooth muscle myocytes and vasodilation. The vasodilatory actions of PDE3 inhibitors contribute to some degree to the hemodynamic responses to these agents, but the primary response that is sought in clinical situations is inotropic rather than vasodilatory.

A large number of clinical trials of PDE3 inhibitors in patients with heart failure have been carried out. Unfortunately, despite short-term inotropic effects, adverse effects on mortality were common. In a recent meta-analysis covering every published clinical trial of PDE3 inhibitors, the long-term administration of these drugs was associated with an ~3%-per-year increase in cardiac mortality that corresponded to a similar increase in the incidence of sudden death (Amsallem et al. 2005) (Fig. 3). The fact that increases in mortality have also been observed in patients treated with β -adrenergic receptor agonists while reductions in mortality have been seen in patients treated with β -adrenergic receptor antagonists has led to

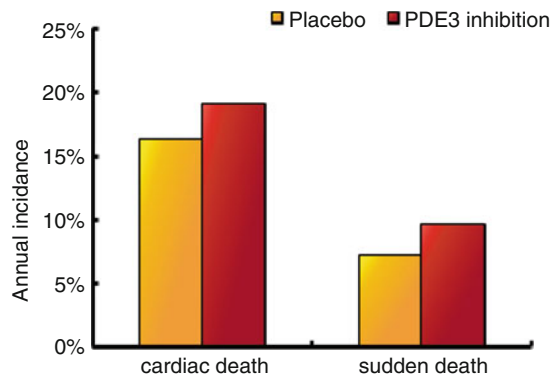


Fig. 3 Mortality in patients treated with PDE3 inhibitors

the understanding that the reduction in cAMP-mediated signaling in failing hearts, despite its negative effect on myocardial contractility, confers some advantages that are forfeited when cAMP-raising therapeutic strategies are implemented (for review, see (Movsesian and Bristow 2005)). Most of the studies showing increased mortality with PDE3 inhibition were carried out before the implantation of cardiac defibrillators in patients with dilated cardiomyopathy became standard therapy, and it is possible that these adverse effects would be less of an issue in patients with defibrillators. In addition, several studies suggest that the concomitant administration of antiarrhythmic agents can improve clinical outcomes in patients receiving long-term PDE3 inhibition, but this has not, to our knowledge, been tested in a large, randomized prospective trial (Nanas et al. 2004; Drakos et al. 2009).

The specific molecular mechanisms to which the inotropic and proarrhythmic consequences of PDE3 inhibition are attributable have not been established with certainty, but inotropic effects are likely to result from increases in the phosphorylation of several membrane-bound substrates of protein kinase A. Phosphorylation of L-type Ca^{2+} channels increases Ca^{2+} influx during systole (Sculptoreanu et al. 1993); phosphorylation of ryanodine-sensitive Ca^{2+} channels increases Ca^{2+} release by the sarcoplasmic reticulum (Takasago et al. 1989); and phosphorylation of phospholamban blocks its inhibitory interaction with SERCA2, the Ca^{2+} -transporting ATPase of the sarcoplasmic reticulum, resulting in an increase in Ca^{2+} accumulation during diastole (Simmerman and Jones 1998). These actions increase the amplitude of intracellular Ca^{2+} transients, which are attenuated in dilated cardiomyopathy (Beuckelmann et al. 1992). Studies in animal models suggest that the phosphorylation of phospholamban may be the most therapeutically relevant of these mechanisms. Depletion of phospholamban and expression of a nonfunctional mutant form of the protein – which mimics the stimulation of SERCA2 activity seen with phospholamban phosphorylation – increase contractility in cultured cardiac myocytes, while germline ablation of phospholamban, knockdown with antisense RNA and expression of anti-phospholamban antibody-derived proteins improve contractile function and prevent pathologic remodeling (Minamisawa et al. 1999; He et al. 1999; Hoshijima et al. 2002; Iwanaga et al. 2004; Eizema et al. 2000; Watanabe et al. 2004; Suckau et al. 2009; Dieterle et al. 2005; Meyer et al. 2004).

Less is known about the molecular mechanisms responsible for the adverse long-term effects of PDE3 inhibition. It is not clear whether the increase in arrhythmias and sudden death observed in clinical trials is due to proarrhythmic consequences of the increase in intracellular Ca^{2+} cycling or to a cAMP-mediated acceleration of pathologic remodeling. The reductions in sudden death seen in clinical trials of angiotensin-converting enzyme inhibitors, isosorbide dinitrate/hydralazine, and β -adrenergic receptor antagonists may be evidence for the latter explanation (Amsallem et al. 2005). Pathologic remodeling has been associated with an increase in apoptosis in human disease and in animal models (for review, see Dorn 2009). Recent studies in rats suggest that PDE3 inhibition has proapoptotic consequences associated with a sustained increase in the expression of inducible cAMP early repressors (“ICERs”), and that this effect is mediated through the phosphorylation of the transcription factor CREB by PKA (Ding et al. 2005a, b; Yan et al. 2007).

3 Altered PDE4 Activity and Heart Failure

While the focus of this chapter is on phosphodiesterase inhibition and heart failure, it is worth noting the possible involvement of another phosphodiesterase in this disease. A leak of sarcoplasmic reticulum-sequestered Ca^{2+} through ryanodine-sensitive Ca^{2+} channels has been identified as a possible pathogenetic mechanism in heart failure in humans, and this leak has been associated with hyperphosphorylation of these channels (Nimer et al. 1995; Reiken et al. 2003; Marx et al. 2000). PDE4D3, a cAMP-specific phosphodiesterase present at relatively low levels in human myocardium, co-immunoprecipitates with ryanodine-sensitive Ca^{2+} channels, and the activity of ryanodine-sensitive Ca^{2+} channel-bound PDE4D3 is significantly decreased in patients with heart failure (Lehnart et al. 2005). This decrease in associated PDE4D3 activity might explain the hyperphosphorylation of the ryanodine-sensitive Ca^{2+} channels. These findings are evidence of a potential adverse effect of PDE4 inhibition in heart failure. The consideration at this point is hypothetical, as PDE4 inhibitors are not used in the treatment of cardiovascular disease.

4 Effects of PDE5 Inhibition in Cardiac Disease

Enzymes in the PDE5 family are characterized by their high selectivity and moderately high affinity for cGMP ($K_M \sim 2.0 \mu\text{M}$) and by their importance in the regulation of vascular smooth muscle contraction. One gene, PDE5A, gives rise to three isoforms, of which two, PDE5A1 and PDE5A2, are expressed in heart (Lin et al. 2002). PDE5 inhibitors such as sildenafil increase intracellular cGMP content in vascular smooth muscle cells, and the resulting potentiation of cGMP-mediated vasodilatory responses is the basis for their use in the treatment of erectile dysfunction and pulmonary hypertension (Francis and Corbin 2005; Kass et al. 2007; Movsesian et al. 2009). These subjects are covered elsewhere in this volume.

More recently, investigators have been studying effects that are more likely attributable to the inhibition of PDE5 activity in cardiac muscle itself (as opposed to effects that can be attributed to the vasodilating effects of PDE5 inhibition). Sildenafil blocks the development of isoproterenol-induced cardiac hypertrophy in rats, an effect that is accompanied by an increase in survival (Hassan and Ketat 2005). Similar observations have been made in mice, where sildenafil blocks and reverses the development of hypertrophy following aortic constriction (Takimoto et al. 2005; Nagayama et al. 2009). Other benefits have been demonstrated in models of myocardial infarction, and sildenafil has been shown to reduce infarct size following ischemic injury in rabbit and mouse models (Ockaili et al. 2002; Salloum et al. 2003). Sildenafil protects against the apoptotic consequences of hypoxia in isolated mouse ventricular myocytes (Das et al. 2005), and antiapoptotic and survival benefits of sildenafil have been demonstrated in mice whose left anterior descending coronary arteries were ligated (Salloum et al. 2008). Finally, sildenafil has been shown to have

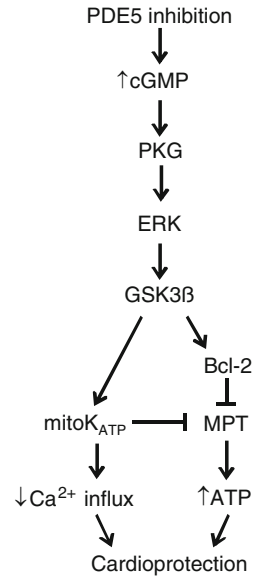
antiapoptotic effects and to protect against the development of contractile dysfunction in a mouse model of doxorubicin toxicity (Fisher et al. 2005). While the mechanisms of these effects in different models cannot be assumed to be identical, they all appear to be dependent upon increases in intracellular cGMP content and the consequent activation of protein kinase G (PKG) (Salloum et al. 2003; Nagayama et al. 2008; Das et al. 2008). Downstream signaling pathways involving calcineurin/NFAT, phosphoinositide-3 kinase (PI3K)/Akt, and ERK have been implicated, and evidence for the role of ERK phosphorylation and the induction of NO synthases and Bcl-2 in the cardioprotective effects following ischemic injury is especially strong (Takimoto et al. 2005; Das et al. 2008, 2009). The vasodilatory action of PDE5 inhibitors could potentially release endogenous mediators of cardioprotection, including adenosine and bradykinin, that trigger a signaling cascade that includes the activation of protein kinase C and generation of NO by phosphorylation of eNOS (Das et al. 2009). NO activates guanylyl cyclase resulting in enhanced formation of cGMP, which activates PKG (Das et al. 2008; Salloum et al. 2009).

One mechanism by which PKG induces cardioprotection is the ERK-dependent opening of mitochondrial K_{ATP} (mito K_{ATP}) channels (Das et al. 2009). Mito K_{ATP} channels feature prominently in the mechanism of cardioprotection because their opening partially depolarizes mitochondrial membranes; this leads to an attenuation of mitochondrial calcium accumulation during ischemia by decreasing the driving force for Ca^{2+} uptake (Liu et al. 1998). Also, it has been shown that sildenafil causes PKG-dependent phosphorylation and inactivation of glycogen synthase kinase 3 β (GSK3 β) in conjunction with an increase in Bcl-2/Bax ratio in cardiac myocytes and in the intact heart (Das et al. 2008). Inhibition of GSK-3 β delays the opening of the mitochondrial permeability transition (MPT) pore, a large-conductance pore in the inner mitochondrial membrane that is opened under conditions associated with ischemia/reperfusion, such as high matrix reactive oxygen species and high matrix calcium (Juhaszova et al. 2004). Mito K_{ATP} opening also inhibits MPT and decreases cell death through mechanism involving activation of mitochondrial PKC- ϵ (Garlid et al. 2009). Moreover, pharmacological inhibitors of MPT have been shown to reduce ischemia/reperfusion injury, suggesting that activation of MPT might have a role in ischemia/reperfusion-mediated cell death (Lim et al. 2007). Thus, the PKG-dependent opening of mito K_{ATP} channels or phosphorylation of GSK-3 β , with subsequent blocking of MPT, may have significant therapeutic implications in protection against ischemia-reperfusion injury with sildenafil and other PDE5 inhibitors (Fig. 4).

5 Possible Involvement of PDE1 Inhibition in the Effects of Sildenafil

The benefits of PDE5 inhibition in mouse models would be impressive if applicable to the treatment of cardiac disease in humans, but there is reason to question whether this is likely. In studies of cyclic nucleotide phosphodiesterases in normal human

Fig. 4 Signaling pathways of PKG-dependent cardioprotection in myocardial ischemia-reperfusion injury



myocardium, PDE5 seemed to be present at low levels compared to other cGMP-hydrolytic activities, though other reports indicated that PDE5 expression increased in pathologic human myocardium (Hambleton et al. 2005; Vandeput et al. 2007; Nagendran et al. 2007; Pokreisz et al. 2009). Recently, the contribution of PDE5 to cGMP-hydrolytic activity in the left ventricles of mice with normal hearts and mice with heart failure as a result of myocardial infarction was compared to its contribution to cGMP-hydrolytic activity in normal and failing human left ventricles. In normal mice, PDE5 comprised ~ 22% of the Ca^{2+} /calmodulin-independent cGMP-hydrolytic activity of the left ventricles; the percentage was ~ 43% in failing mouse hearts. In left ventricular myocardium from normal and failing human hearts, in contrast, PDE5 comprised $\leq 5\%$ of the Ca^{2+} /calmodulin-independent cGMP-hydrolytic activity (Vandeput et al. 2009). This large difference in the relative levels of PDE5 in mouse and human myocardium raises some doubt as to whether PDE5 inhibition is as likely to be as beneficial in humans as in mouse models.

At the same time, while sildenafil is generally regarded as PDE5-selective, its potency as an inhibitor of PDE5 is only ~30-fold higher than its potency as an inhibitor of PDE1 (Vandeput et al. 2009). Enzymes in the latter family are characterized by their activation by Ca^{2+} and calmodulin and their moderately high affinity for cGMP and, for some isoforms, cAMP (Bender 2007). PDE1 is present in high abundance in human left ventricular myocardium (Fig. 1) and in mouse left ventricular myocardium. In several of the studies showing cardioprotective effects in mouse hearts, sildenafil was used at concentrations $\geq 1.0 \mu\text{M}$ (Nagendran et al. 2007; Das et al. 2005; Fisher et al. 2005; Pokreisz et al. 2009; Takimoto et al. 2007), and at this concentration its inhibition of cGMP-hydrolytic activity in mouse left ventricles is

attributable to inhibition of both PDE5 and PDE1 (Vandeput et al. 2009). This finding would suggest that inhibition of PDE1 may have contributed to sildenafil's potentiation of cGMP-mediated signaling and its consequent cardioprotective actions in animal models. A recent study in mouse models has, in fact, shown that PDE1 inhibition was effective in blocking phenylephrine-induced hypertrophy of cardiac myocytes in vitro and isoproterenol-induced hypertrophy in vivo (Miller et al. 2009). These effects were additive with those of sildenafil, raising the possibility that the antihypertrophic effects of PDE5 inhibition and of PDE1 inhibition involve different mechanisms.

6 Conclusions

The role for phosphodiesterase inhibitors in the treatment of cardiac muscle disease is unclear. The use of PDE3 inhibition to raise intracellular cAMP content is a widely accepted approach to acute decompensation in patients with heart failure, but its usefulness as an option for long-term therapy is controversial. The large number of studies whose results, taken together, show an increase in sudden death in patients treated with PDE3 inhibitors have led most clinicians to try to avoid the chronic use of these agents and most pharmaceutical companies to "pass" on investing in new approaches to PDE3 inhibition.

At this time, there is probably much more interest in the cardioprotective actions of inhibiting cGMP-hydrolytic activity in cardiac myocytes. The benefits seen with sildenafil in animal models of cardiac disease are very impressive, and there is growing insight into the downstream mechanisms that are involved. The low abundance of PDE5 in human myocardium relative to the animal models is a cause for some concern regarding the therapeutic potential of PDE5 inhibition in cardiac muscle disease, at least with regard to direct myocardial actions. The results of ongoing clinical trials of sildenafil in humans with heart failure are likely to provide greater insight. The possibility that the benefits of potentiating cGMP-mediated signaling demonstrated in these animal models might alternatively be achieved through inhibition of other cGMP-hydrolytic enzymes, especially PDE1, is a very new concept that warrants further investigation.

References

- Amsallem E, Kasparian C, Haddour G, Boissel JP, Nony P (2005) Phosphodiesterase III inhibitors for heart failure. *Cochrane Database Syst Rev* 25:CD002230
- Baim DS, McDowell AV, Cherniles J et al (1983) Evaluation of a new bipyridine inotropic agent – milrinone – in patients with severe congestive heart failure. *N Engl J Med* 309:748–756
- Bender AT (2007) Calmodulin-stimulated cyclic nucleotide phosphodiesterases. In: Beavo JA, Francis SH, Houslay MD (eds) *Cyclic nucleotide phosphodiesterases in health and disease*. CRC Press, Boca Raton, FL, pp 35–54

- Beuckelmann DJ, Nabauer M, Erdmann E (1992) Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. *Circulation* 85:1046–1055
- Bristow MR, Ginsburg R, Umans V et al (1986) Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure. *Circ Res* 59:297–309
- Choi YH, Ekholm D, Krall J et al (2001) Identification of a novel isoform of the cyclic-nucleotide phosphodiesterase PDE3A expressed in vascular smooth-muscle myocytes. *Biochem J* 353:41–50
- Das A, Xi L, Kukreja RC (2005) Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. Essential role of nitric oxide signaling. *J Biol Chem* 280:12944–12955
- Das A, Xi L, Kukreja RC (2008) Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. *J Biol Chem* 283:29572–29585
- Das A, Salloum FN, Xi L, Rao YJ, Kukreja RC (2009) ERK phosphorylation mediates sildenafil-induced myocardial protection against ischemia-reperfusion injury in mice. *Am J Physiol Heart Circ Physiol* 296:H1236–H1243
- Dieterle T, Meyer M, Gu Y et al (2005) Gene transfer of a phospholamban-targeted antibody improves calcium handling and cardiac function in heart failure. *Cardiovasc Res* 67:678–688
- Ding B, Abe J, Wei H et al (2005a) Functional role of phosphodiesterase 3 in cardiomyocyte apoptosis: implication in heart failure. *Circulation* 111:2469–2476
- Ding B, Abe J, Wei H et al (2005b) A positive feedback loop of phosphodiesterase 3 (PDE3) and inducible cAMP early repressor (ICER) leads to cardiomyocyte apoptosis. *Proc Natl Acad Sci USA* 102:14771–14776
- Dorn GW 2nd (2009) Apoptotic and non-apoptotic programmed cardiomyocyte death in ventricular remodelling. *Cardiovasc Res* 81:465–473
- Drakos SG, Kanakakis JV, Nanas S et al (2009) Intermittent inotropic infusions combined with prophylactic oral amiodarone for patients with decompensated end-stage heart failure. *J Cardiovasc Pharmacol* 53:157–161
- Eizema K, Fechner H, Bezstarosti K et al (2000) Adenovirus-based phospholamban antisense expression as a novel approach to improve cardiac contractile dysfunction: comparison of a constitutive viral versus an endothelin-1-responsive cardiac promoter. *Circulation* 101:2193–2199
- Fisher PW, Salloum F, Das A, Hyder H, Kukreja RC (2005) Phosphodiesterase-5 inhibition with sildenafil attenuates cardiomyocyte apoptosis and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity. *Circulation* 111:1601–1610
- Frace AM, Mery PF, Fischmeister R, Hartzell HC (1993) Rate-limiting steps in the beta-adrenergic stimulation of cardiac calcium current. *J Gen Physiol* 101:337–353
- Francis SH, Corbin JD (2005) Sildenafil: efficacy, safety, tolerability and mechanism of action in treating erectile dysfunction. *Expert Opin Drug Metab Toxicol* 1:283–293
- Garlid KD, Costa AD, Quinlan CL, Pierre SV, Dos Santos P (2009) Cardioprotective signaling to mitochondria. *J Mol Cell Cardiol* 46:858–866
- Hambleton R, Krall J, Tikishvili E et al (2005) Isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP hydrolytic activity in subcellular fractions of human myocardium. *J Biol Chem* 280:39168–39174
- Han SJ, Vaccari S, Nedachi T et al (2006) Protein kinase B/Akt phosphorylation of PDE3A and its role in mammalian oocyte maturation. *EMBO J* 25:5716–5725
- Hassan MA, Ketat AF (2005) Sildenafil citrate increases myocardial cGMP content in rat heart, decreases its hypertrophic response to isoproterenol and decreases myocardial leak of creatine kinase and troponin T. *BMC Pharmacol* 5:10
- He H, Meyer M, Martin JL et al (1999) Effects of mutant and antisense RNA of phospholamban on SR Ca(2+)-ATPase activity and cardiac myocyte contractility. *Circulation* 100:974–980

- Hoshijima M, Ikeda Y, Iwanaga Y et al (2002) Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. *Nat Med* 8:864–871
- Iwanaga Y, Hoshijima M, Gu Y et al (2004) Chronic phospholamban inhibition prevents progressive cardiac dysfunction and pathological remodeling after infarction in rats. *J Clin Invest* 113:727–736
- Jaski BE, Fifer MA, Wright RF, Braunwald E, Colucci WS (1985) Positive inotropic and vasodilator actions of milrinone in patients with severe congestive heart failure. Dose-response relationships and comparison to nitroprusside. *J Clin Invest* 75:643–649
- Juhaszova M, Zorov DB, Kim SH et al (2004) Glycogen synthase kinase-3 β mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. *J Clin Invest* 113:1535–1549
- Kass DA, Champion HC, Beavo JA (2007) Phosphodiesterase type 5: expanding roles in cardiovascular regulation. *Circ Res* 101:1084–1095
- Kenan Y, Murata T, Shakur Y, Degerman E, Manganiello VC (2000) Functions of the N-terminal region of cyclic nucleotide phosphodiesterase 3 (PDE 3) isoforms. *J Biol Chem* 275:12331–12338
- Lehnart SE, Wehrens XH, Reiken S et al (2005) Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. *Cell* 123:25–35
- Lim SY, Davidson SM, Hausenloy DJ, Yellon DM (2007) Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. *Cardiovasc Res* 75:530–535
- Lin CS, Chow S, Lau A, Tu R, Lue TF (2002) Human PDE5A gene encodes three PDE5 isoforms from two alternate promoters. *Int J Impot Res* 14:15–24
- Liu Y, Sato T, O'Rourke B, Marban E (1998) Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation* 97:2463–2469
- Marx SO, Reiken S, Hisamatsu Y et al (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* 101:365–376
- Meyer M, Belke DD, Trost SU et al (2004) A recombinant antibody increases cardiac contractility by mimicking phospholamban phosphorylation. *FASEB J* 18:1312–1314
- Miller CL, Oikawa M, Cai Y et al (2009) Role of Ca²⁺/calmodulin-stimulated cyclic nucleotide phosphodiesterase 1 in mediating cardiomyocyte hypertrophy. *Circ Res* 105:956–964
- Minamisawa S, Hoshijima M, Chu G et al (1999) Chronic phospholamban-sarcoplasmic reticulum calcium ATPase interaction is the critical calcium cycling defect in dilated cardiomyopathy. *Cell* 99:313–322
- Movsesian MA, Bristow MR (2005) Alterations in cAMP-mediated signaling and their role in the pathophysiology of dilated cardiomyopathy. *Curr Top Dev Biol* 68:25–48
- Movsesian MA, Smith CJ, Krall J, Bristow MR, Manganiello VC (1991) Sarcoplasmic reticulum-associated cyclic adenosine 5'-monophosphate phosphodiesterase activity in normal and failing human hearts. *J Clin Invest* 88:15–19
- Movsesian M, Stehlik J, Vandeput F, Bristow MR (2009) Phosphodiesterase inhibition in heart failure. *Heart Fail Rev* 14:255–263
- Nagayama T, Zhang M, Hsu S, Takimoto E, Kass DA (2008) Sustained soluble guanylate cyclase stimulation offsets nitric-oxide synthase inhibition to restore acute cardiac modulation by sildenafil. *J Pharmacol Exp Ther* 326:380–387
- Nagayama T, Hsu S, Zhang M et al (2009) Sildenafil stops progressive chamber, cellular, and molecular remodeling and improves calcium handling and function in hearts with pre-existing advanced hypertrophy caused by pressure overload. *J Am Coll Cardiol* 53:207–215
- Nagendran J, Archer SL, Soliman D et al (2007) Phosphodiesterase type 5 is highly expressed in the hypertrophied human right ventricle, and acute inhibition of phosphodiesterase type 5 improves contractility. *Circulation* 116:238–248
- Nanas JN, Tsagalou EP, Kanakakis J et al (2004) Long-term intermittent dobutamine infusion, combined with oral amiodarone for end-stage heart failure: a randomized double-blind study. *Chest* 125:1198–1204

- Nimer LR, Needleman DH, Hamilton SL, Krall J, Movsesian MA (1995) Effect of ryanodine on sarcoplasmic reticulum Ca²⁺ accumulation in nonfailing and failing human myocardium. *Circulation* 92:2504–2510
- Ockaili R, Salloum F, Hawkins J, Kukreja RC (2002) Sildenafil (Viagra) induces powerful cardioprotective effect via opening of mitochondrial K(ATP) channels in rabbits. *Am J Physiol Heart Circ Physiol* 283:H1263–H1269
- Pokreisz P, Vandewijngaert S, Bito V et al (2009) Ventricular phosphodiesterase-5 expression is increased in patients with advanced heart failure and contributes to adverse ventricular remodeling after myocardial infarction in mice. *Circulation* 119:408–416
- Reiken S, Gaburjakova M, Guatimosim S et al (2003) Protein kinase A phosphorylation of the cardiac calcium release channel (ryanodine receptor) in normal and failing hearts: Role of phosphatases and response to isoproterenol. *J Biol Chem* 278:444–453
- Salloum F, Yin C, Xi L, Kukreja RC (2003) Sildenafil induces delayed preconditioning through inducible nitric oxide synthase-dependent pathway in mouse heart. *Circ Res* 92:595–597
- Salloum FN, Abbate A, Das A et al (2008) Sildenafil (Viagra) attenuates ischemic cardiomyopathy and improves left ventricular function in mice. *Am J Physiol Heart Circ Physiol* 294: H1398–H1406
- Salloum FN, Chau VQ, Hoke NN et al (2009) Phosphodiesterase-5 inhibitor, tadalafil, protects against myocardial ischemia/reperfusion through protein-kinase g-dependent generation of hydrogen sulfide. *Circulation* 120:S31–S36
- Sculptoreanu A, Rotman E, Takahashi M, Scheuer T, Catterall WA (1993) Voltage-dependent potentiation of the activity of cardiac L-type calcium channel alpha 1 subunits due to phosphorylation by cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 90:10135–10139
- Shakur Y, Takeda K, Kenan Y et al (2000) Membrane localization of cyclic nucleotide phosphodiesterase 3 (PDE3). Two N-terminal domains are required for the efficient targeting to, and association of, PDE3 with endoplasmic reticulum. *J Biol Chem* 275:38749–38761
- Shimizu K, Shintani Y, Ding WG, Matsuura H, Bamba T (2002) Potentiation of slow component of delayed rectifier K(+) current by cGMP via two distinct mechanisms: inhibition of phosphodiesterase 3 and activation of protein kinase G. *Br J Pharmacol* 137:127–137
- Simmerman HK, Jones LR (1998) Phospholamban: protein structure, mechanism of action, and role in cardiac function. *Physiol Rev* 78:921–947
- Sinoway LS, Maskin CS, Chadwick B, Forman R, Sonnenblick EH, Le Jemtel TH (1983) Long-term therapy with a new cardiotoxic agent, WIN 47203: drug-dependent improvement in cardiac performance and progression of the underlying disease. *J Am Coll Cardiol* 2:327–331
- Suckau L, Fechner H, Chemaly E et al (2009) Long-term cardiac-targeted RNA interference for the treatment of heart failure restores cardiac function and reduces pathological hypertrophy. *Circulation* 119:1241–1252
- Takasago T, Imagawa T, Shigekawa M (1989) Phosphorylation of the cardiac ryanodine receptor by cAMP-dependent protein kinase. *J Biochem (Tokyo)* 106:872–877
- Takimoto E, Champion HC, Li M et al (2005) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* 11:214–222
- Takimoto E, Belardi D, Tocchetti CG et al (2007) Compartmentalization of cardiac beta-adrenergic inotropy modulation by phosphodiesterase type 5. *Circulation* 115:2159–2167
- Uretsky BF, Generalovich T, Reddy PS, Spangenberg RB, Follansbee WP (1983) The acute hemodynamic effects of a new agent, MDL 17, 043, in the treatment of congestive heart failure. *Circulation* 67:823–828
- Vandecasteele G, Verde I, Rucker-Martin C, Donzeau-Gouge P, Fischmeister R (2001) Cyclic GMP regulation of the L-type Ca(2+) channel current in human atrial myocytes. *J Physiol* 533:329–340
- Vandeput F, Wolda SL, Krall J et al (2007) Cyclic nucleotide phosphodiesterase PDE1C1 in human cardiac myocytes. *J Biol Chem* 282:32749–32757
- Vandeput F, Krall J, Ockaili R et al (2009) cGMP-hydrolytic activity and its inhibition by sildenafil in normal and failing human and mouse myocardium. *J Pharmacol Exp Ther* 330:884–891

- Watanabe A, Arai M, Yamazaki M, Koitabashi N, Wuytack F, Kurabayashi M (2004) Phospholamban ablation by RNA interference increases Ca^{2+} uptake into rat cardiac myocyte sarcoplasmic reticulum. *J Mol Cell Cardiol* 37:691–698
- Wechsler J, Choi YH, Krall J, Ahmad F, Manganiello VC, Movsesian MA (2002) Isoforms of cyclic nucleotide phosphodiesterase PDE3A in cardiac myocytes. *J Biol Chem* 277:38072–38078
- Yan C, Miller CL, Abe J (2007) Regulation of phosphodiesterase 3 and inducible cAMP early repressor in the heart. *Circ Res* 100:489–501

Phosphodiesterases: Emerging Therapeutic Targets for Neonatal Pulmonary Hypertension

Kathryn N. Farrow and Robin H. Steinhorn

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Abstract Pulmonary hypertension in the neonate is associated with multiple underlying problems such as respiratory distress syndrome, meconium aspiration syndrome, congenital diaphragmatic hernia, bronchopulmonary dysplasia, sepsis, or congenital heart disease. Because of the heterogeneous group of disorders, the therapeutic approach and response often depends on the underlying disease. In many of these conditions, there is evidence that cyclic nucleotide signaling and specifically phosphodiesterases (PDEs) are disrupted. PDE inhibitors represent an emerging class of pulmonary vasodilators in adults. Studies are now under way to evaluate the utility, efficacy, and safety of such therapies in infants with pulmonary hypertension.

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Abbreviations

BPD	Bronchopulmonary dysplasia
CDH	Congenital diaphragmatic hernia
COX	Cyclooxygenase
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FPASM	Fetal pulmonary artery smooth muscle cells
H ₂ O ₂	Hydrogen peroxide
iNOS	Inducible nitric oxide synthase
iNO	Inhaled nitric oxide
MAS	Meconium aspiration syndrome
NAC	<i>N</i> -acetyl-cysteine
NSAIDs	Nonsteroidal anti-inflammatory drugs
PPHN	Persistent pulmonary hypertension of the newborn
PDE	Phosphodiesterase
PGIS	Prostacyclin synthase
ROS	Reactive oxygen species
RVH	Right ventricular hypertrophy
sGC	Soluble guanylate cyclase

1 Phosphodiesterases in the Pathophysiology of Neonatal Pulmonary Hypertension

1.1 Normal Pulmonary Vascular Transition

Pulmonary hypertension is normal during fetal life. Because the placenta, not the lung, serves as the organ of gas exchange, only 10% of the cardiac output is circulated through the pulmonary vascular bed. In utero, pulmonary pressures are equivalent to systemic pressures due to elevated pulmonary vascular resistance. As gestation and fetal lung growth progress, rapid vascular growth increases the number of small pulmonary arteries within the lung by tenfold, preparing the lungs to accept the dramatic increase in blood flow that occurs at birth (Levin et al. 1976). Despite this increase in vascular surface area, pulmonary vascular resistance actually increases with gestational age when corrected for lung or body weight. These findings indicate that vascular constriction must play a key role in maintaining high pulmonary vascular tone during fetal life. There are multiple pathways involved in maintaining high pulmonary vascular tone in utero. Some of the known pulmonary

vasoconstrictors include hypoxia (a defining feature of fetal development), as well as endothelin-1 (ET-1), thromboxane, acidosis, and various mediators of inflammation (Lakshminrusimha and Steinhorn 1999).

As gestation progresses, the mediators of pulmonary vasodilation become more dominant. In particular, pulmonary expression of endothelial nitric oxide (NO) synthase (eNOS), inducible NO synthase (iNOS), and NO production increase near the time of birth (Abman et al. 1990; Shaul et al. 2002). Coincident increases in soluble guanylate cyclase (sGC) activity promote increased vascular cGMP, which then leads to vasorelaxation via decreasing intracellular calcium (Bloch et al. 1997). Another potentially important vasodilatory pathway in the fetal lung is the prostacyclin pathway. Cyclooxygenase (COX) is the rate-limiting enzyme that generates prostacyclin from arachadonic acid. COX-1 in particular is upregulated in late gestation (Brannon et al. 1994, 1998), leading to an increase in prostacyclin production in late gestation and early postnatal life (Brannon et al. 1994; Leffler et al. 1984). Prostacyclin upregulates adenylyl cyclase to increase intracellular cAMP levels, which then lead to vasorelaxation.

Phosphodiesterases (PDEs) counteract these cAMP and cGMP vasodilatory pathways, and as described extensively within this text, they comprise a superfamily of enzymes that includes 11 different PDE families with specific tissue and cellular distributions (Conti and Beavo 2007; Lugnier 2006). The prevalent PDE within the lung is PDE5, although there are significant amounts of PDE1, PDE3, and PDE4 as well (Maclean et al. 1997). PDE5, a cGMP-specific PDE, was initially characterized in bovine lung and has since been found in multiple other tissues (Loughney et al. 1998). In rats, PDE5 expression and activity steadily increase through the end of gestation, peak on day of life one, and then drop dramatically into adulthood, strongly suggesting developmental regulation (Sanchez et al. 1998). In contrast, in neonatal lambs, PDE5 activity and expression appear to acutely decrease within 1 h after birth and then rise again at 4–7 days of life (Farrow et al. 2008a; Hanson et al. 1998a; Okogbule-Wonodi et al. 1998; Sanchez et al. 1998). Furthermore, PDE5 activity is higher in pulmonary arteries than pulmonary veins in lambs, suggesting location-dependent as well as developmental regulation (Okogbule-Wonodi et al. 1998). As the primary enzyme responsible for regulating cGMP, PDE5 potentially represents the most important regulator of NO-mediated vascular relaxation in the normal pulmonary vascular transition after birth (Abman et al. 1990; Lakshminrusimha and Steinhorn 1999).

Unlike PDE5, less is known about the developmental regulation of other cGMP PDEs in the fetal and neonatal lung. PDE1, a dual specificity PDE, consists of three isoforms in mammals; PDE1A and PDE1B have higher affinity for cGMP but hydrolyze both cyclic nucleotides with similar efficacy. In contrast, PDE1C hydrolyzes cGMP and cAMP with equal affinity and rate. All three isoforms of PDE1 have been described in the pulmonary vasculature of various animals and in human pulmonary artery smooth muscle cells (Evgenov et al. 2006). While their presence has been documented in older animals and adult humans, there has been little study of PDE1 expression or activity in the perinatal period. A recent study in neonatal mice indicates that PDE1A mRNA decreased postnatally in normoxia, but

was increased by exposure to hyperoxia for 21 days, although PDE1A protein expression remained unchanged (Woyda et al. 2009). Thus, regulation of PDE1 in the neonatal pulmonary vasculature is unclear, and further investigation is needed.

As described above, prostacyclin–cAMP signaling operates in parallel to the NO–cGMP pathway for perinatal pulmonary vasodilation and is regulated in part by cAMP-hydrolyzing PDEs such as PDE3 and PDE4. Our group recently published that PDE3A expression and activity in the resistance pulmonary arteries increase dramatically by 24 h after birth. These results were surprising and unexpected, as we would have predicted that similar to PDE5, PDE3 activity would decrease after birth to facilitate increased cAMP levels (Chen et al. 2009). This increase may be acting to establish cAMP-containing regulatory regions within the pulmonary vascular smooth muscle cell after birth, although it is unclear what role PDE3 has in normal pulmonary vascular transition after birth.

Even less is known about the perinatal regulation of PDE4, which has strong specificity for cAMP hydrolysis. PDE4 in the lung has been most extensively studied in the airway smooth muscle (Fan Chung 2006). However, more recent studies have demonstrated the presence of PDE4 in adult human pulmonary artery smooth muscle cells and that exposure to hypoxia increases expression of several PDE4 isoforms without impacting total PDE4 activity (Millen et al. 2006). Thus, while no one has specifically examined PDE4 around the time of birth, it is plausible to hypothesize that PDE4 might be differentially regulated between the relatively hypoxic in utero environment and the normoxic extra-uterine environment.

At birth, a rapid and dramatic decrease in pulmonary vascular resistance allows half of the combined ventricular output to be redirected from the placenta to the lung, leading to an eight- to tenfold increase in pulmonary blood flow. The stimuli that seem to be most important are lung inflation with a gas, a decrease in carbon dioxide tension, and an increase in oxygen tension. Each of these stimuli will independently decrease PVR and increase pulmonary blood flow, with the largest effects seen when the two events occur simultaneously. For instance, oxygen directly and indirectly stimulates the activity of both eNOS and COX-1 immediately after birth, leading to increased levels of the vasodilators, NO and prostacyclin (Shaul et al. 1992; Shaul and Wells 1994; Steinhorn et al. 1994). Shear stress is also known to regulate the synthesis of NO in the fetal circulation. During transition, the initial increase in pulmonary blood flow in response to ventilation or oxygenation likely leads to increased shear stress in the vasculature, which further potentiates NO production (Uematsu et al. 1995). In contrast, PDE5 expression and activity fall after birth in the pulmonary vasculature, further accentuating upstream effects leading to increased cGMP and vasodilation (Farrow et al. 2008a; Sanchez et al. 1998). It is unclear which specific signals cause PDE5 to fall as part of the normal transition. However, if events in utero and at the time of birth impair these critical transition steps, they may lead to elevated pulmonary pressures and the symptomatic infant with persistent pulmonary hypertension of the newborn (PPHN).

1.2 Pathophysiology of Persistent Pulmonary Hypertension of the Newborn

When the normal cardiopulmonary transition fails to occur, the result is PPHN. PPHN describes a syndrome characterized by common pathophysiologic features including sustained elevation of pulmonary vascular resistance and hypoxemia due to right-to-left extrapulmonary shunting of blood flow across the ductus arteriosus or foramen ovale. PPHN affects 2–6 per 1,000 live births or approximately 10% of all infants admitted to neonatal intensive care and is accompanied by an 8–10% risk of death and significant short-term and long-term morbidity (Walsh-Sukys et al. 2000). The physiologic findings of PPHN may be found in association with a wide range of cardiopulmonary disorders such as meconium aspiration, sepsis, pneumonia, asphyxia, congenital diaphragmatic hernia (CDH), respiratory distress syndrome, and others. Pathological findings include pulmonary vascular remodeling and smooth muscle hyperplasia, often in the absence of significant lung parenchyma pathology (Haworth 1988; Murphy et al. 1981). PPHN can largely be thought of as one of three types: (1) the abnormally constricted pulmonary vasculature, which is the most common type and includes diagnoses such as meconium aspiration syndrome (MAS), respiratory distress syndrome, and sepsis; (2) the structurally abnormal vasculature, which is often termed idiopathic PPHN; or (3) the hypoplastic vasculature such as is seen in CDH or alveolar capillary dysplasia, a rare malformation of lung development. The pathophysiology of each type is dependent on the point in gestation when the normal transition to extrauterine life fails. Thus, since the underlying pathophysiology differs, different pulmonary vasodilators may be more or less successful for treatment and a thorough understanding of the pathways that are disrupted in each condition will be key to determine the most appropriate therapy.

1.2.1 Meconium Aspiration Syndrome

The most common cause of PPHN is meconium aspiration syndrome (MAS), which affects 25,000–30,000 infants with 1,000 deaths annually in the United States (Gelfand et al. 2004). In these cases, the infant passes meconium while still in utero, usually in response to stressful stimuli. Affected infants aspirate the meconium into their airways, where it can impede ventilation, cause severe pneumonitis, and induce lung inflammatory changes. While a fair bit is known about the parenchymal disease associated with MAS, there is much less known about the utility of specific pulmonary vasodilators. In the NINOS trial, 51% of the infants had MAS as the underlying cause of their PPHN. In that study, treatment with inhaled nitric oxide (iNO) decreased the combined outcome of death or cardiopulmonary bypass support (known as ECMO), suggesting that modulation of the NO–cGMP pathway may be a useful treatment modality for infants with MAS (Group 1997). However, little data exist to address whether the PDEs are

specifically dysregulated in MAS. Interestingly, a recent study demonstrated that sildenafil is a potent pulmonary vasodilator in a neonatal piglet model of MAS (Shekerdemian et al. 2002, 2004).

1.2.2 Idiopathic PPHN

Idiopathic PPHN is the second most common etiology of PPHN and is classically described in near-term (>34 weeks gestation) and term newborns (Konduri 2004; Walsh-Sukys et al. 2000). Autopsy studies of fatal PPHN demonstrate severe hypertensive structural remodeling with vessel wall thickening and smooth muscle hyperplasia even in newborns who die shortly after birth, suggesting that many cases of severe disease are associated with chronic intrauterine stress. In these patients, the vascular smooth muscle extends to the level of the intra-acinar arteries, which does not normally occur until much later in the postnatal period (Haworth 1988; Murphy et al. 1981). The severity of vascular remodeling does not allow the pulmonary vasculature in these infants to appropriately vasodilate in response to birth-related stimuli, and they will present with profound hypoxemia and clear lung fields on X-ray, leading many to refer to this as “black-lung” PPHN (Farrow et al. 2005; Konduri 2004; Lakshminrusimha and Steinhorn 1999).

Because idiopathic PPHN is characterized by “pure” vascular disease, it is probably the best studied of the various causes of neonatal pulmonary hypertension, and many groups have worked to characterize the underlying fetal and neonatal pathophysiology that might lead to abnormal remodeling of the fetal and early neonatal pulmonary vasculature. For instance, a well-known cause of idiopathic PPHN is constriction of the fetal ductus arteriosus in utero from exposure to nonsteroidal anti-inflammatory drugs (NSAIDs) during the third trimester (Manchester et al. 1976). A fetal lamb model was developed by surgically closing the ductus in utero, which induces rapid increases in fetal pulmonary artery pressure, pulmonary vascular remodeling, and a subsequent failure to transition to extrauterine life (Morin 1989; Wild et al. 1989).

Decreased expression and activity of eNOS have been documented in infants with PPHN, as well as in the ductal-ligation lamb model (Shaul et al. 1997; Villanueva et al. 1998). Increased production of reactive oxygen species (ROS) by multiple sources leads to vasoconstriction, smooth muscle hypertrophy, and NOS dysfunction (Brennan et al. 2003; Konduri et al. 2003). Further, activity of vascular sGC is diminished in PPHN lambs (Steinhorn et al. 1995), and activity of PDE5 is increased (Hanson et al. 1998b), both of which lead to decreased cGMP concentrations (Tzao et al. 2001).

While prostacyclin appears to be important in the normal pulmonary vascular transition, much less is known about potential dysregulation of the prostacyclin pathway in PPHN (Konduri 2004; Lakshminrusimha et al. 2009a). Our group has recently demonstrated decreased expression of the prostacyclin synthase (PGIS) and the prostacyclin IP receptor in PPHN lambs, leading to reduced vasodilation to

prostacyclin analogues. Pretreatment with milrinone, a PDE3 inhibitor, restores relaxations to prostanoids to levels similar to that seen in control fetuses. While fetal levels of PDE3 are not altered by PPHN, PPHN suppresses the normal rise in PDE3 expression and activity following delivery and mechanical ventilation (Chen et al. 2009; Lakshminrusimha et al. 2009a). Interestingly, nitric oxide increases PDE3 expression and activity in these lambs, demonstrating the interrelated nature of cGMP and cAMP pathways in PPHN.

Because neonatal PPHN is associated with severe hypoxemia, the use of high oxygen concentrations, up to 100% oxygen, is typically considered as a first-line therapy in infants with PPHN (Farrow et al. 2005; Tiktinsky and Morin 1993). However, the use of oxygen may greatly exaggerate oxidative stress in multiple cellular compartments of the diseased vasculature. Recent data suggest that hyperoxia may diminish vascular responses to endogenous and exogenous nitric oxide in both the normal and remodeled pulmonary vasculature, indicating that ROS may inactivate NO or other enzymes responsible for mediating its vascular effects (Lakshminrusimha et al. 2007a, 2009b). The mechanisms by which ROS lead to pulmonary vasoconstriction and diminished NO responsiveness is certainly complicated, but emerging data indicate multiple targets in the pulmonary vascular regulatory pathways, including the PDEs. We recently published that exposing normal fetal pulmonary artery smooth muscle cells (FPASM) to hyperoxia for 24 h leads to decreased cGMP response to exogenous NO. We further demonstrated that exposure to hyperoxia for 24 h increases PDE5 mRNA and protein expression as well as increased level of PDE5 phosphorylation and activity. We also noted a dose–response effect of hyperoxia on PDE5 activity, with a stepwise increase noted in PDE5 activity as the cells were treated with 21, 50, and 95% O₂ (Fig. 1). Inhibition of the hyperoxia-induced PDE5 activity with sildenafil was sufficient to partially rescue the cGMP response to exogenous NO, further indicating that PDE5 is a critical regulator of cGMP in the context of hyperoxia (Farrow et al. 2008a).

As might be expected, exposure to hyperoxia for 24 h led to increased oxidative stress within the FPASM. As such, we hypothesized that ROS may serve as critical mediators in the crosstalk between oxygen and PDE5. In support of that, a single dose of an exogenous oxidant, hydrogen peroxide (H₂O₂), was sufficient to induce long-lasting changes in PDE5 expression, phosphorylation, and activity, which mirrored those seen after exposure to hyperoxia. Similarly, the changes in PDE5 expression and activity as well as the decreased cGMP responsiveness in hyperoxia were all reversed with pretreatment with a chemical antioxidant, *N*-acetyl-cysteine (NAC). This confirms that ROS, in general, and H₂O₂, in particular, are sufficient to induce significant increases in PDE5 expression and activity in the pulmonary artery smooth muscle cell, which may promote vasoconstriction, poor NO response, and vascular remodeling (Farrow et al. 2008a).

In order to better understand the impact of ROS on the intact neonatal pulmonary vasculature, we recently ventilated both healthy control and PPHN lambs immediately after birth with 100% oxygen, in order to simulate the clinical scenario seen with a human infant with severe PPHN. Resuscitation of healthy lambs with 100% oxygen significantly exaggerated pulmonary arterial contractile responses

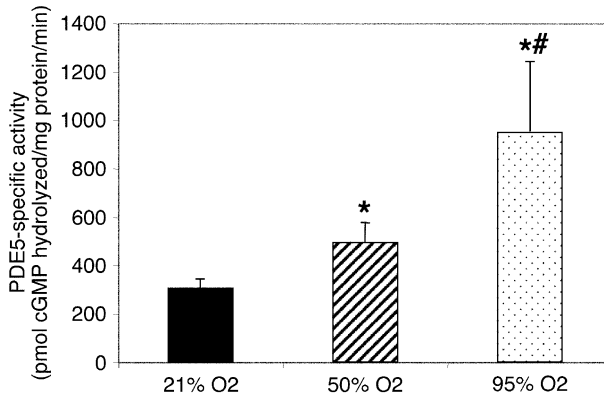


Fig. 1 Hyperoxia increases PDE5 activity in Ovine FPASMCs. FPASMCs were exposed to 21% O₂-5% CO₂, 50% O₂-5% CO₂, or 95% O₂-5% CO₂ for 24 h, and total protein was harvested. PDE5-specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total milligrams of protein (200 μ M cGMP substrate in assay; 100 nM sildenafil for inhibition). Data are shown as mean \pm SEM ($n = 8$; read in duplicate). * $P < 0.05$ vs. 21% O₂, # $P < 0.05$ vs. 50% O₂. Reproduced with permission from Farrow et al. (2008a)

to norepinephrine compared to vessels from animals resuscitated with 21% oxygen (Lakshminrusimha et al. 2006). Use of 100% oxygen ventilation significantly increased pulmonary vascular PDE5 expression and activity (Fig. 2) (Farrow et al. 2008a), indicating that PDE5 activity is an important mediator in the vascular response to oxygen and hyperoxia. More recently, we reported that ventilation of PPHN lambs with 100% oxygen results in pulmonary vasoconstriction and diminished pulmonary vascular relaxation to inhaled NO (Lakshminrusimha et al. 2009b). Very recent studies indicate that these abnormal responses are mediated in part by a greatly exaggerated increase in PDE5 activity (Farrow et al. 2010a, b).

Ventilation of healthy control lambs with either 21 or 100% oxygen blunts the increase in PDE3 expression and activity normally seen in the healthy spontaneously breathing 1-day lambs, suggesting that PDE3 may be dysregulated by mechanical forces associated with ventilation rather than by hyperoxia (Chen et al. 2009). This dysregulation of PDE3 expression and activity may lead to a disruption of the normal cAMP signaling within the pulmonary vascular smooth muscle during this critical transition time. Recent studies have shown that the PDE3 inhibitor milrinone, when used in conjunction with either iNO or iloprost, can help to restore normal vascular responses (Chen et al. 2009; Lakshminrusimha et al. 2009a).

Thus, idiopathic PPHN is a complex pathological process induced by disruption of multiple signaling pathways, including the NO-cGMP-PDE5 and prostacyclin-cAMP-PDE3 pathways, leading to vasoconstriction and structural remodeling of the vasculature. Postnatal treatment with high levels of oxygen exacerbates these abnormalities, leading to decreases in cGMP and cAMP and impaired vasodilation. Inhibitors of PDE5 and PDE3, such as sildenafil and

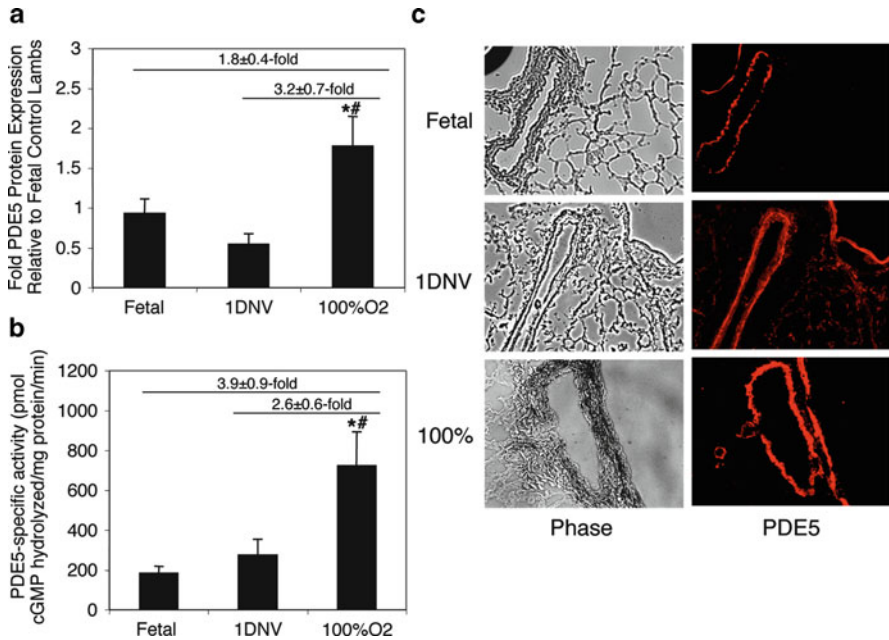


Fig. 2 Ventilation of healthy neonatal sheep with 100% O₂ induces PDE5 protein expression and activity. Ovine lung parenchyma and resistance PAs from healthy lambs ventilated with 100% O₂ were harvested after 24 h and compared with both fetal lambs (Fetus) and healthy 1-day nonventilated lambs (1DNV). **(a)** PA PDE5 protein expression was analyzed via Western blot, with β-actin normalization. Data are shown as mean ± SEM. **P* < 0.05 vs. fetal lambs, #*P* < 0.05 vs. 1-day nonventilated lambs. **(b)** Lung PDE5-specific activity was measured as the sildenafil-inhibitable fraction of total cGMP hydrolysis, normalized for total milligrams of protein (200 μM cGMP substrate in assay; 100 nM sildenafil for inhibition). Data are shown as mean ± SEM. **P* < 0.05 vs. fetal lambs, #*P* < 0.05 vs. 1-day nonventilated lambs. **(c)** PDE5 expression is localized within the PA smooth muscle and nearby airways. The *left column* shows phase-contrast images (×10) of frozen lamb lung sections. The *right column* shows the corresponding sections stained for PDE5, with immunofluorescence shown as *red*. Reproduced with permission from Farrow et al. (2008a)

milrinone, represent novel and particularly attractive new therapies for these infants (Fig. 3) (Lakshminrusimha and Steinhorn 2009).

1.2.3 Congenital Diaphragmatic Hernia

CDH is a serious birth defect that includes disordered development of the diaphragm and a variable degree of pulmonary hypoplasia. It occurs in 1 of every 2,000–4,000 live births and accounts for 8% of all major congenital anomalies. Severe CDH develops early in the course of lung development, and as a result airway divisions and alveolarization may be significantly impaired. Because development of the pulmonary arterial system parallels development of the bronchial tree, fewer arterial

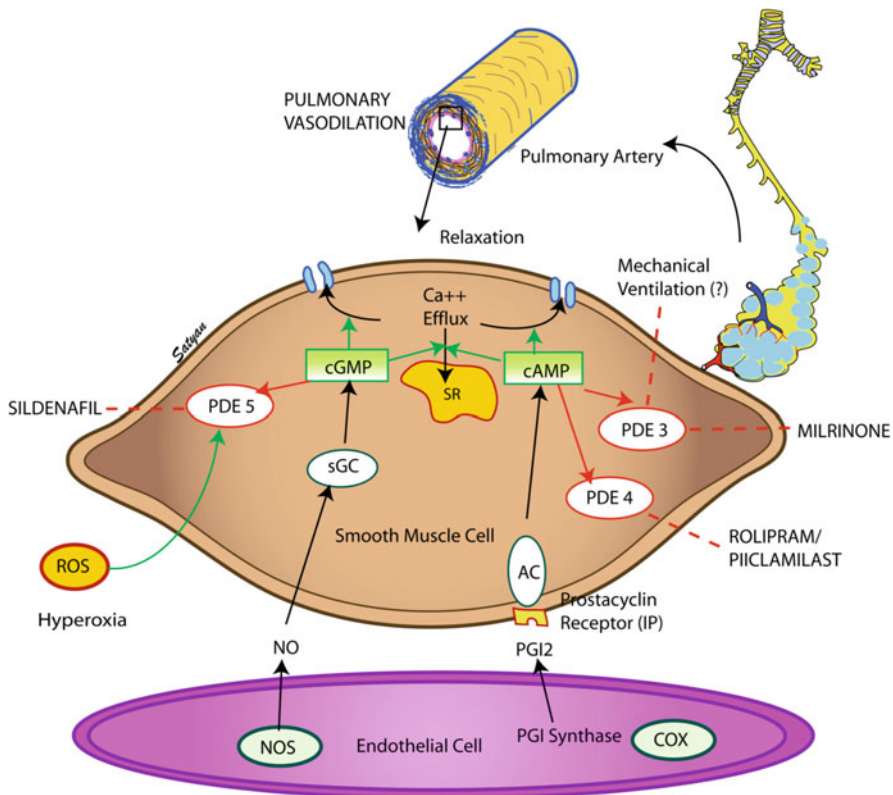


Fig. 3 Mechanism of action of phosphodiesterase inhibitors in PPHN. PDEs play a critical role in regulating cGMP and cAMP within the vascular smooth muscle. Elevations in cGMP and cAMP lead to vasorelaxation by decreasing intracellular calcium via decreasing calcium efflux from the sarcoplasmic reticulum and increasing calcium efflux from the cell. PDEs hydrolyze cGMP and cAMP, thereby preventing cyclic nucleotide-mediated vasorelaxation. Thus, PDE inhibitors such as sildenafil and milrinone represent promising therapies for neonates with PPHN resistant to traditional therapy with inhaled nitric oxide. *Dashed lines* represent inhibition. Figure kindly provided by Dr. Satyan Lakshminrusimha

branches are observed in CDH. Further, abnormal medial muscular hypertrophy is observed as far distally as the acinar arterioles. Pulmonary capillary blood flow is decreased because of the small cross-sectional area of the pulmonary vascular bed, and flow may be further decreased by abnormal pulmonary vasoconstriction (de Buys Roessingh and Dinh-Xuan 2009; Farrow et al. 2005).

There is no ideal animal model in which the disruption in the diaphragm occurs at the same stage of lung development as is seen in human fetuses. The most widely utilized is the nitrofen-induced rat model of CDH. In this model, reduced lung eNOS activity and expression has been described, thereby pinpointing the eNOS–cGMP pathway as important in the pathogenesis of CDH and its accompanying pulmonary hypertension (Karamanoukian et al. 1996). Another group has

shown that response to exogenous nitric oxide and a cGMP analogue, 8-Br-cGMP, is impaired in resistance pulmonary arterioles of the CDH animals. Of note, pretreatment of CDH resistance pulmonary arterioles with the PDE5 inhibitor zaprinast completely restored their vasodilatory response to 8-Br-cGMP (Vukcevic et al. 2005), suggesting that PDE5 may also be involved with the pathogenesis of pulmonary hypertension in the infant with CDH.

1.3 Pathophysiology of Pulmonary Hypertension in the Older Infant

There are two other major classes of infants who develop pulmonary hypertension – former preterm infants who develop pulmonary hypertension as a result of their underlying lung disease and infants, both term and preterm, who develop pulmonary hypertension as a result of their congenital heart disease. In both cases, the disease process begins during the neonatal period, but symptoms may not become manifest until the infant is several months old. Further, the resulting pulmonary hypertension is not only a disease of altered vasoreactivity, but it also involves significant vascular remodeling. The role of PDEs in the pathophysiology of these infants is just beginning to be elucidated.

1.3.1 Bronchopulmonary Dysplasia and Cor Pulmonale

Bronchopulmonary dysplasia (BPD) is a common complication of preterm birth that affects approximately 30% of infants (or roughly 10,000 babies per year) with extreme prematurity (typically defined as a birthweight <1,000 g). BPD results in significant long-term morbidity in childhood, including poor lung function, diminished growth, and impaired neurodevelopment (Jobe and Bancalari 2001; Walsh et al. 2006). A recently recognized complication of moderate or severe BPD is pulmonary hypertension and right-sided heart failure, or cor pulmonale. While the overall risk of BPD clearly correlates with gestational age and birthweight, it remains unclear why some infants develop mild or severe disease. Even less is known about why some infants develop pulmonary hypertension and how to appropriately treat these infants. Poor outcomes, including mortality, are distressingly common (Jobe and Bancalari 2001; Khemani et al. 2007; Mourani et al. 2008).

Recent clinical trials have laid the foundation for the use of iNO for prevention of BPD (Ballard et al. 2006; Kinsella et al. 2006). Smaller case series also suggest that the use of iNO may also improve oxygenation in those BPD infants with established pulmonary hypertension (Mourani et al. 2008). Additionally, premature babies are forced to live in an environment with higher oxygen concentrations than would be encountered during fetal development. Not surprisingly, emerging evidence indicates that oxidative stress may produce significant lung parenchymal and

vascular injury (Farrow et al. 2008a, b, 2010a, b; Lakshminrusimha et al. 2006, 2007a, b; Thebaud et al. 2005; Vento et al. 2001). As discussed above, PDE5 expression and function may be adversely affected by ROS-mediated events (Farrow et al. 2008a, 2010a, b).

Consistent with this hypothesis, three studies address the role of cGMP and PDE5 in models of preterm lung disease – one study in preterm lambs and two studies in neonatal rats. In the preterm lamb model of BPD, treatment with a cGMP analogue that is not hydrolyzable by PDE5 decreased pulmonary vascular resistance, which would indirectly suggest that PDE5 plays a role in the elevated pulmonary vascular resistance seen with BPD (Bland et al. 2003). Perhaps more intriguing are the results seen in the rat model of BPD, induced by hyperoxia. In that model, treatment of the neonatal rats with sildenafil resulted in decreased pulmonary vascular resistance, decreased right ventricular hypertrophy (RVH), and decreased medial wall thickness of pulmonary arteries. This study also showed that sildenafil improved lung alveolarization, suggesting that PDE5 may play a critical role in both alveolar and vascular development (Fig. 4) (Ladha et al. 2005). A more recent study in the rat model extends these studies and demonstrated that treatment with sildenafil prior to hyperoxia exposure increased lung cGMP levels and improved survival as well as lung alveolarization and angiogenesis. In another arm of that study, rescue treatment with sildenafil (administration of drug 6 days after initiation of hyperoxia) significantly decreased pulmonary vessel medial wall thickness and reduced RVH (de Visser et al. 2009).

Interesting new data have recently emerged implicating a role for PDE4 in the pathogenesis of BPD. In one study (de Visser et al. 2008), preterm rats were exposed to room air, hyperoxia, or hyperoxia with either of the PDE4 inhibitors, rolipram or piclamilast (Houslay et al. 2005). PDE4 inhibition prolonged median survival and decreased lung inflammation and vascular leakage as well as decreased markers of inflammation (de Visser et al. 2008). Another model of BPD, produced by prolonged exposure of mice to hyperoxia, results in decreased alveolarization and increased septal wall thickness (Woyda et al. 2009). Pretreatment with a PDE4 inhibitor decreased septal wall thickness and increased total airspace area, suggesting that PDE4 may be of critical importance in neonatal lung development (Woyda et al. 2009).

1.3.2 Congenital Heart Disease

Congenital heart disease, particularly that associated with increased pulmonary arterial blood flow from left-to-right shunts, increased right-sided pressures from nonrestrictive septal defects and aorto-pulmonary shunts, and increased pulmonary venous congestion, can lead to remodeling of the pulmonary vascular bed with associated pulmonary hypertension (Hoffman et al. 1981). The risk of developing pulmonary hypertension in these patients is dependent on many factors including the age of the patient, the type of congenital heart disease, and the degree of pulmonary overcirculation. Endothelial dysfunction has been hypothesized to

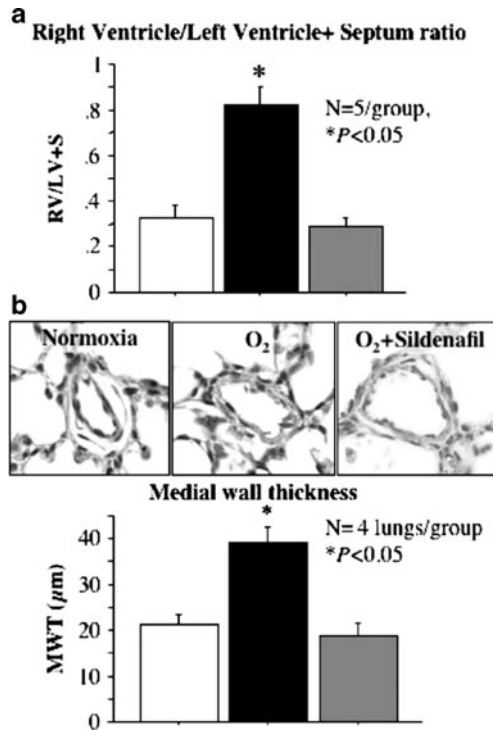


Fig. 4 Sildenafil reduces pulmonary hypertension associated with oxygen-induced BPD. (a) Right ventricular hypertrophy. Hyperoxia-exposed rats had significant right ventricular hypertrophy (RVH) as indicated by the increase in RV/LV+S ratio compared with normoxic controls. Sildenafil treatment reduced RVH (sildenafil 100 mg/kg subcutaneously daily during hyperoxia exposure). (b) Medial wall thickness. A representative picture of pulmonary arteries is shown displaying a thickened medial arterial wall in hyperoxic rat lungs as compared with normoxic controls. Sildenafil treatment significantly reduced the % medial wall thickness as compared with untreated hyperoxic pulmonary arteries (sildenafil 100 mg/kg subcutaneously daily during hyperoxia exposure). Reprinted with permission of the American Thoracic Society. Copyright American Thoracic Society; Ladha et al. (2005); Official Journal of the American Thoracic Society, Diane Gern, Publisher

play a major role in this vascular dysfunction and remodeling. Adult patients with pulmonary hypertension have impaired endothelium-dependent pulmonary vasodilation and decreased eNOS expression (Giaid and Saleh 1995). Children with pulmonary hypertension due to congenital heart disease are also thought to have endothelial dysfunction, and they have also been described to have extension of smooth muscle cells to peripheral pulmonary arteries, neointimal formation due to smooth muscle cell migration, medial hypertrophy, and plexigenic lesion formation (Rabinovitch et al. 1978, 1984, 1986).

A number of studies in a juvenile lamb model of pulmonary hypertension due to chronic pulmonary overcirculation have indicated its pathophysiology includes

abnormal NO–cGMP–PDE5 signaling and excess production of ROS and endothelin (Black et al. 1998, 2000, 2001, 2003; Lakshminrusimha et al. 2007b; Steinhorn et al. 2001). In this model (Reddy et al. 1996), an aortopulmonary shunt is placed in fetal lambs during the last week of gestation, followed by spontaneous delivery. The lambs have impaired endothelium-dependent vasorelaxation by 4 weeks of age (Reddy et al. 1996), associated with complex vascular changes including increased pulmonary vascular eNOS expression and downstream derangements such as increased pulmonary sGC protein expression and increased PDE5 protein expression and activity (Black et al. 1998, 2001). Further studies demonstrate that antioxidants such as superoxide dismutase normalized vascular reactivity in the shunt vessels and that the ROS in these lambs likely are the result of uncoupled NOS (Lakshminrusimha et al. 2007b; Steinhorn et al. 2001).

Other studies have explored the impact of various treatment strategies on the NO–cGMP–PDE5 pathway in lambs with congenital heart disease. Inhaled NO treatment of shunted lambs decreases lung eNOS expression without changing NOS activity, decreases sGC protein expression, and has no effect on PDE5 protein expression (Ross et al. 2005). However, acute withdrawal of the iNO rapidly and dramatically increases pulmonary vascular resistance by 45% and decreases in cGMP levels (Ross et al. 2005). These findings would suggest that the PDE5 in these lambs becomes the dominant determinant of cGMP concentration after withdrawal of iNO (Ross et al. 2005). An additional study attempted to address the role of cAMP in rebound pulmonary hypertension upon iNO withdrawal (Thelitz et al. 2004). Treatment of shunt lambs with iNO for 24 h decreased lung cAMP concentrations, both during iNO treatment and upon acute withdrawal of iNO. Concomitant treatment with milrinone, a PDE3 inhibitor, normalized cAMP concentrations during iNO treatment and upon acute withdrawal. Milrinone also prevented the increase in pulmonary vascular resistance seen upon acute iNO withdrawal (Thelitz et al. 2004).

The increased pulmonary vascular resistance seen in these shunted lambs upon withdrawal of iNO is consistent with the well-known phenomenon of rebound pulmonary hypertension seen in human infants upon acute withdrawal of iNO (Atz et al. 1996). Taken together, these studies suggest that iNO may alter expression and activity of PDEs, such as PDE3 and PDE5, and that inhibitors of PDE3 and PDE5 may be of clinical utility to treat rebound pulmonary hypertension upon iNO withdrawal (Ross et al. 2005; Thelitz et al. 2004).

2 PDE Inhibitors in Neonatal Pulmonary Hypertension

The initial treatment of the neonate with pulmonary hypertension depends in part on the underlying disorder. Therapy often includes aggressive support of cardiac function and perfusion with volume and inotropic agents to enhance cardiac output and systemic O₂ transport. While most infants require mechanical ventilation to allow for lung recruitment, an important goal is to avoid postnatal lung injury, which worsens the degree of pulmonary hypertension (Farrow et al. 2005).

When supportive treatment is insufficient, the only FDA-approved pulmonary vasodilator in neonates is iNO. Multicenter randomized, placebo-controlled, blinded trials of iNO in term and near-term infants with PPHN demonstrated that iNO significantly decreased the need for ECMO in newborns with PPHN (Clark et al. 2000; Group 1997). However, up to 40% of infants do not respond or sustain their response to iNO, and it does not reduce mortality, duration of mechanical ventilation, or length of hospitalization. Follow-up studies to 12–24 months also show that iNO does not significantly decrease the incidence of chronic lung disease or adverse neurodevelopmental sequelae (Clark et al. 2003; Group 2000).

In addition to the problem of inadequate response to iNO, many infants will acutely develop “rebound” pulmonary hypertension upon its discontinuation (Atz et al. 1996; Farrow et al. 2005). As noted above, this finding was first described in young infants receiving inhaled NO after surgery for congenital heart disease, although the phenomenon frequently occurs in infants with PPHN as well. Rebound pulmonary hypertension tends to be most severe if discontinuation occurs when infants are breathing higher concentrations of iNO, but may also occur even after more gradual iNO weaning. It is important for clinicians to understand that rebound pulmonary hypertension can occur even in infants who were apparent nonresponders to iNO therapy, particularly after treatment has been delivered for more than an hour. While rebound pulmonary hypertension typically responds to reinstatement of iNO, it represents a significant limitation of this therapy (Atz et al. 1996; Farrow et al. 2005).

Additional pulmonary vasodilators are urgently needed for infants with pulmonary hypertension. Inhibition of PDEs represents a potentially powerful and novel way to achieve safe, sustained, and even selective pulmonary vasodilation in these infants.

2.1 PDE5 Inhibitors

PDE5 is by far the best studied of the PDEs for involvement in the pathophysiology of neonatal pulmonary hypertension. Its dysregulation has been implicated in most of the major causes of neonatal pulmonary hypertension including PPHN, CDH, BPD, and congenital heart disease. The PDE5 inhibitor sildenafil has been shown to be an effective treatment in adult pulmonary arterial hypertension (Galie et al. 2005) and is now marketed as Revatio for this purpose. Not surprisingly, PDE5 inhibitors have been among the first PDE inhibitors used in neonates. While many have proposed their use to augment iNO therapy, there is some evidence to suggest that PDE5 inhibitors may serve as effective vasodilators in their own right.

2.1.1 Dipyridamole

One of the earliest drugs used to manipulate the pulmonary circulation was the nonspecific PDE5 inhibitor dipyridamole (Persantin) (al-Alaiyan et al. 1996;

Dukarm et al. 1998; Ivy et al. 1998). In neonatal lambs with PPHN, dipyridamole significantly decreased pulmonary vascular resistance and pulmonary blood pressure, and increased pulmonary blood flow. Unfortunately, it was not selective for the pulmonary vasculature, and it decreased systemic blood pressure to a similar degree (Dukarm et al. 1998). In human neonates, there are a handful of case reports reporting the use of dipyridamole to facilitate iNO weaning in infants with PPHN, congenital heart disease, and CDH. In two case reports, treatment with dipyridamole facilitated iNO weaning in infants with PPHN, and in both cases, the infants survived (al-Alaiyan et al. 1996; Worwag et al. 2000). In infants with CDH, successful treatment with dipyridamole has been reported, but improvement was transient in at least two cases (Buysse et al. 2001; Thebaud et al. 1999). The largest report of dipyridamole use was in infants who required postoperative iNO therapy for pulmonary hypertension after repair of congenital heart disease. In a series of 23 consecutive children receiving iNO postoperatively, seven developed rebound pulmonary hypertension when iNO was acutely stopped. In those seven, dipyridamole attenuated the rise in pulmonary pressure after acute withdrawal of iNO (Ivy et al. 1998). None of these case reports described a significant decrease in systemic blood pressure similar to that observed in the PPHN lamb model (Dukarm et al. 2005).

2.1.2 Sildenafil

Sildenafil has been demonstrated to acutely decrease pulmonary vascular resistance and improve pulmonary blood flow in a neonatal porcine model of MAS (Shekerdemian et al. 2002, 2004). Chronic use of sildenafil decreased medial wall thickness and RVH in a rat model of BPD (Ladha et al. 2005). Like dipyridamole, one of the earliest clinical uses of sildenafil was to facilitate weaning from iNO in infants with congenital heart disease following corrective surgery. In an initial case series, enteral sildenafil increased circulating cGMP and allowed two of three infants to wean from iNO without rebound pulmonary hypertension (Atz and Wessel 1999). Two subsequent studies have expanded these initial observations. Oral sildenafil facilitated iNO discontinuation in seven infants (ages 3 days to 21 months) who had previously failed to wean from iNO (Lee et al. 2008). Namachivayam et al. delivered enteral sildenafil ($n = 15$) or placebo ($n = 14$) to a total of 29 infants with critical illness (most with congenital heart disease), who were breathing 2 ppm iNO in preparation for a discontinuation trial. Ten out of 14 patients (71%) receiving placebo experienced rebound pulmonary hypertension after iNO was stopped. In contrast, administration of a single dose of oral sildenafil (0.4 mg/kg) prevented rebound pulmonary hypertension in all 15 treated patients. The authors also noted a significant reduction in the duration of mechanical ventilation and ICU length of stay in the group that received sildenafil (Namachivayam et al. 2006).

Some data raise specific concerns regarding the use of sildenafil in combination with iNO. In piglets with meconium aspiration, intravenous sildenafil in combination with iNO enhanced the decrease in pulmonary artery pressure and pulmonary

vascular resistance, but also produced systemic hypotension and decreased oxygenation (Shekerdemian et al. 2004). Similarly, in neonates after repair of ventricular or atrioventricular septal defects, systemic hypotension and decreased oxygenation were noted when sildenafil and iNO were given concurrently (Stocker et al. 2003). It is possible that sildenafil in combination with iNO may increase pulmonary perfusion to underventilated lung segments, thereby leading to worsening VQ mismatch and decreasing oxygenation. Furthermore, when given intravenously, sildenafil may not be selective to the pulmonary vascular bed. It is possible that a drop in systemic blood pressure may be poorly tolerated if combined with diminished oxygenation due to worsened VQ mismatch. We have shown that idiopathic PPHN is associated with increased PDE5 expression and activity in the pulmonary vascular bed (Farrow et al. 2008a, 2010a, b). Thus, those infants may be better able to tolerate the combined therapy of iNO and intravenous sildenafil.

The clinical use of sildenafil has recently been reported in infants with PPHN, including one small, randomized controlled trial with oral sildenafil, and one pilot pharmacokinetic, pharmacodynamic trial with intravenous sildenafil (Baquero et al. 2006; Steinhorn et al. 2009). As mentioned previously, the only FDA-approved pulmonary vasodilator for infants is iNO. However, iNO therapy is currently expensive and unavailable in many parts of the world, leading to much higher mortality from PPHN in those regions. For this reason, the use of oral sildenafil as the primary treatment for severe PPHN was studied in term and near-term infants in Colombia. This study randomized 13 infants (>35 5/7 weeks gestation and age <3 days) with an oxygenation index greater than 25, indicating severe PPHN. In the sildenafil treatment group, a significant improvement in oxygenation was observed by 24 h and six of the seven infants survived. In contrast, the infants in the placebo group did not improve their oxygenation and only one of the six infants survived (Baquero et al. 2006). Despite these encouraging results, significant concerns exist regarding giving sildenafil enterally to infants who may have compromised intestinal perfusion with unknown and inconsistent rates of gastrointestinal absorption.

Two recently published papers described the results of a multicenter pilot trial examining the safety and pharmacokinetics of intravenous sildenafil in term and near-term neonates with PPHN (Mukherjee et al. 2009; Steinhorn et al. 2009). Thirty-six neonates (>34 weeks gestation) were enrolled at a mean age of 34 ± 17 h when they had moderate to severe pulmonary hypertension with an oxygenation index >15 (Mukherjee et al. 2009; Steinhorn et al. 2009). Infants with congenital anomalies including CDH were excluded. Infants received IV sildenafil administered within eight progressive, “step-up” dosing groups, and the study also determined the optimal loading dose and duration. Infants received sildenafil for a minimum of 48 h. After that time, sildenafil was discontinued once infants had been successfully weaned from iNO for at least 1 h, or after 7 days (168 h). Based on data from adults, clearance via the hepatic CYP3A4 and CYP2C9 enzymes was expected. In the study, sildenafil clearance in neonates increased threefold between day of life one and the end of the first week of life, likely reflecting postnatal maturation of this CYP system. Hence, postnatal age at the time of sildenafil

initiation has a significant impact on drug clearance. Similarly, significant increases in sildenafil concentration should be expected when it is used concomitantly with CYP3A4 inhibitors such as cimetidine and erythromycin (Mukherjee et al. 2009).

Hypotension was the most commonly observed adverse effect. One infant was withdrawn from the study because the sildenafil loading infusion (scheduled to be given over 5 min) had to be stopped after 2 min due to systemic hypotension. Hypotension was not observed when the loading dose was delivered over 3 h. An additional infant died due to bilateral tension pneumothoraces, not related to sildenafil infusion. For the remaining 34 infants, oxygenation improved significantly through the course of the sildenafil infusion, with no significant changes in systemic blood pressure. Only one infant required cannulation for extracorporeal life support. Of the seven infants enrolled without prior use of iNO, all experienced a significant improvement in oxygenation within 4 h after sildenafil administration (Fig. 5). Only one of the seven infants required iNO, and the other six infants improved and survived to hospital discharge without requiring either iNO or ECMO (Steinhorn et al. 2009). Unlike the previously mentioned studies, sildenafil used in combination with iNO did not significantly decrease systemic blood pressure or

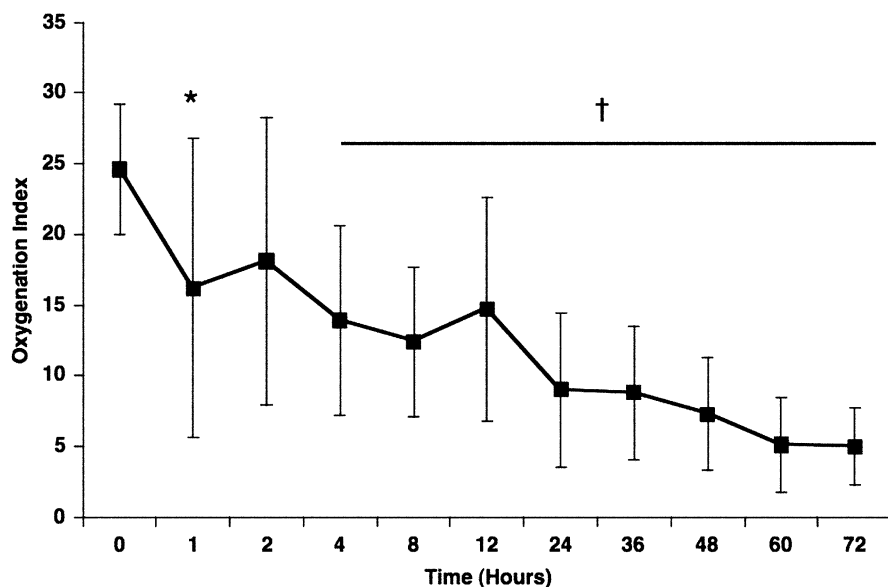


Fig. 5 Response to sildenafil intravenous infusion without iNO. Seven infants were enrolled before the need for iNO. Oxygenation index (OI) was improved by 1 h (24.6 ± 4.6 to 16.1 ± 9.9 ; $*P = 0.0502$), with significant and sustained improvement by 4 h after the initiation of sildenafil (14.7 ± 6.4 , $p = 0.0088$). Of the seven infants who received sildenafil only, the majority ($n = 5$) were in cohorts 6–8, meaning that they received the highest maintenance infusion dose of 1.64 mg/kg/day, and differed only in the approach to the loading dose. Of these seven infants, only one infant required additional treatment with iNO, and that infant was in cohort 4, receiving loading and maintenance doses that were approximately 20% of the final dose tested in trial. Reprinted from Steinhorn et al. (2009) with permission from Elsevier

oxygenation. We speculate that it is because there is increased PDE5 expression and activity in the pulmonary vessels of infants with PPHN (Farrow et al. 2008a, 2010a, b). Future studies are planned to further evaluate efficacy of IV sildenafil in neonates with PPHN. Of note, the intravenous form of sildenafil was FDA-approved in December of 2009 for adults with pulmonary arterial hypertension.

Smaller case reports and case series have examined the efficacy of oral sildenafil in infants with CDH and BPD. In one case report, oral sildenafil facilitated weaning from iNO in a 7-week-old infant with CDH, although the infant ultimately died (Keller et al. 2004). In another series of seven infants with CDH, oral sildenafil improved right cardiac output and decreased pulmonary vascular resistance as measured by echocardiography, and five of the seven infants survived (Noori et al. 2007). A larger, double-blind, randomized controlled trial of oral sildenafil in infants with CDH and severe pulmonary hypertension is ongoing (NCT00133679). Infants are recruited between 10 and 42 days of age if they continue to have a significant FiO_2 requirement, if they need ECMO beyond 10 days of age, or if they have a pulmonary artery pressure $>2/3$ systemic pressure beyond 14 days of age. Target enrollment is 32 infants, who will receive placebo or sildenafil for 5 weeks. The primary outcome is pulmonary artery pressure on echocardiogram at the end of the treatment period.

Sildenafil is an attractive therapeutic option for infants with chronic pulmonary hypertension because of its relative ease of administration and apparent low toxicity. A recent case series examined the effect of oral sildenafil in 25 infants and children with pulmonary hypertension due to chronic lung disease, who initiated sildenafil treatment at <2 years of age (14–673 days). Most patients (88%) achieved hemodynamic improvement after a median treatment interval of 40 days, and the majority of infants receiving iNO could be weaned off. Five patients died after initiation of sildenafil treatment, but none died from refractory pulmonary hypertension or right heart failure. This important pilot study suggests that sildenafil is safe for infants with pulmonary hypertension due to chronic lung disease and indicates that further studies are warranted (Mourani et al. 2009). Because sildenafil improved lung alveolarization in neonatal rats, a small double-blind randomized controlled trial of oral sildenafil in the prevention of BPD is ongoing (NCT00431418). The target population is extremely preterm infants <28 weeks gestation who still require ventilatory support at 7 days of age. This study should also provide important initial data about the safety of sildenafil in young preterm infants.

2.2 PDE3 Inhibitors

The PDE3 inhibitor milrinone is most commonly used in pediatric patients for its inotropic effects (Chang et al. 1995; Hoffman et al. 2003; Zaccolo and Movsesian 2007). However, by raising cAMP in the pulmonary vasculature, milrinone can potentially act as a pulmonary vasodilator and improve oxygenation in patients with poor response to iNO. In neonatal lambs with PPHN, one of the unexpected results

of iNO was a dramatic increase in PDE3 activity to levels similar to those observed in normal 1-day lambs. Further, milrinone was found to significantly relax pulmonary arteries isolated from PPHN lambs ventilated with iNO (Chen et al. 2009). In infants with PPHN, there have been two small case series of treatment with milrinone. In both series, the infants were already receiving iNO at the time that the milrinone was started, and significant improvements in oxygenation were observed with milrinone treatment (Bassler et al. 2006; McNamara et al. 2006). Similarly, in pediatric patients ($n = 46$, average age 5 years) undergoing a Fontan procedure, postoperative use of milrinone in conjunction with iNO resulted in greater improvements in the transpulmonary gradient and arterial oxygen saturation versus those children treated with iNO alone or milrinone alone (Cai et al. 2008a, b). These clinical case series all support an interesting potential role for milrinone in the treatment of neonatal pulmonary hypertension, especially when used in conjunction with iNO. However, more study is needed to determine optimum dosing and timing of therapy initiation for milrinone in these infants.

2.3 PDE1 and PDE4 Inhibitors

To date, there have been no trials or case reports of PDE1 or PDE4 inhibitors in neonates with pulmonary hypertension. However, data from adult patients and animal models would suggest that this should be an area of investigation for future potential therapies. For instance, zaprinast, initially thought to be a selective PDE5 inhibitor, has recently been described to have significant inhibitory activity against PDE1. This agent was found to enhance relaxations to iNO in neonatal lambs with PPHN (Thusu et al. 1995). Increased PDE1C mRNA and protein have been described in resistance pulmonary arteries and pulmonary artery smooth muscle cells derived from lung specimens of human pulmonary arterial hypertension patients (Murray et al. 2007; Schermuly et al. 2007). Thus, a PDE1C inhibitor might have clinical utility across all of the different types of neonatal pulmonary hypertension. Similarly, PDE4 inhibitors have reached clinical trials in adults with asthma and chronic obstructive pulmonary disease (Fan Chung 2006). In light of the recent publications that PDE4 inhibition is beneficial in neonatal mice with BPD, it is reasonable to hypothesize that PDE4 inhibitors might be clinically useful in the patients with pulmonary hypertension due to BPD.

3 Conclusions

Neonatal pulmonary hypertension is a clinical syndrome that affects approximately 10% of all infants admitted to neonatal intensive care units. It has multiple etiologies that involve complex alterations in multiple signaling pathways. As such, no one therapy is likely to be appropriate or effective in every patient.

There is growing evidence from vascular smooth muscle cells and various animal models that PDEs play a critically important role in regulating pulmonary vascular tone in the neonatal period. Two early clinical trials of sildenafil have been completed in infants with PPHN with encouraging results, and double-blind randomized controlled trials are under way to assess the effectiveness of sildenafil in CDH and BPD. There is new clinical evidence to suggest that milrinone may be useful in patients with neonatal pulmonary hypertension, particularly when used in conjunction with inhaled NO. However, more data are needed from both preclinical and clinical studies to know how to select the best combination of PDE inhibitors for specific disease states and age groups. Until such studies have been conducted and evaluated, the use of PDE inhibitors for neonatal pulmonary hypertension should be confined to appropriately designed clinical trials (Shah and Ohlsson 2007).

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References

- Abman SH, Chatfield BA, Hall SL, McMurtry IF (1990) Role of endothelium-derived relaxing factor during transition of pulmonary circulation at birth. *Am J Physiol* 259:H1921–H1927
- al-Alaiyan S, al-Omran A, Dyer D (1996) The use of phosphodiesterase inhibitor (dipyridamole) to wean from inhaled nitric oxide. *Intensive Care Med* 22(10):1093–1095
- Atz AM, Wessel DL (1999) Sildenafil ameliorates effects of inhaled nitric oxide withdrawal. *Anesthesiology* 91(1):307–310
- Atz AM, Adatia I, Wessel DL (1996) Rebound pulmonary hypertension after inhalation of nitric oxide. *Ann Thorac Surg* 62(6):1759–1764
- Ballard RA, Truog WE, Cnaan A, Martin RJ, Ballard PL, Merrill JD, Walsh MC, Durand DJ, Mayock DE, Eichenwald EC, Null DR, Hudak ML, Puri AR, Golombek SG, Courtney SE, Stewart DL, Welty SE, Phibbs RH, Hibbs AM, Luan X, Wadlinger SR, Asselin JM, Coburn CE (2006) Inhaled nitric oxide in preterm infants undergoing mechanical ventilation. *N Engl J Med* 355(4):343–353
- Baquero H, Soliz A, Neira F, Venegas ME, Sola A (2006) Oral sildenafil in infants with persistent pulmonary hypertension of the newborn: a pilot randomized blinded study. *Pediatrics* 117(4):1077–1083
- Bassler D, Choong K, McNamara P, Kirpalani H (2006) Neonatal persistent pulmonary hypertension treated with milrinone: four case reports. *Biol Neonate* 89(1):1–5
- Black SM, Fineman JR, Steinhorn RH, Bristow J, Soifer SJ (1998) Increased endothelial NOS in lambs with increased pulmonary blood flow and pulmonary hypertension. *Am J Physiol* 275(5 Pt 2):H1643–H1651
- Black SM, Bekker JM, Johengen MJ, Parry AJ, Soifer SJ, Fineman JR (2000) Altered regulation of the ET-1 cascade in lambs with increased pulmonary blood flow and pulmonary hypertension. *Pediatr Res* 47:97–106
- Black SM, Sanchez LS, Mata-Greenwood E, Bekker JM, Steinhorn RH, Fineman JR (2001) sGC and PDE5 are elevated in lambs with increased pulmonary blood flow and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 281(5):L1051–L1057
- Black SM, Mata-Greenwood E, Dettman RW, Ovadia B, Fitzgerald RK, Reinhartz O, Thelitz S, Steinhorn RH, Gerrets R, Hendricks-Munoz K, Ross GA, Bekker JM, Johengen MJ,

- Fineman JR (2003) Emergence of smooth muscle cell endothelin B-vasoconstriction in lambs with experimental congenital heart disease and increased pulmonary blood flow. *Circulation* 108:1646–1654
- Bland RD, Ling CY, Albertine KH, Carlton DP, MacRitchie AJ, Day RW, Dahl MJ (2003) Pulmonary vascular dysfunction in preterm lambs with chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* 285(1):L76–L85
- Bloch KD, Filippov G, Sanchez LS, Nakane M, de la Monte SM (1997) Pulmonary soluble guanylate cyclase, a nitric oxide receptor, is increased during the perinatal period. *Am J Physiol* 272:L400–L406
- Brannon TS, North AJ, Wells LB, Shaul PW (1994) Prostacyclin synthesis in ovine pulmonary artery is developmentally regulated by changes in cyclooxygenase-1 gene expression. *J Clin Invest* 93:2230–2235
- Brannon TS, MacRitchie AN, Jaramillo MA, Sherman TS, Yuhanna IS, Margraf LR, Shaul PW (1998) Ontogeny of cyclooxygenase-1 and cyclooxygenase-2 gene expression in ovine lung. *Am J Physiol* 274:L66–L71
- Brennan LA, Steinhorn RH, Wedgwood S, Mata-Greenwood E, Roark EA, Russell JA, Black SM (2003) Increased superoxide generation is associated with pulmonary hypertension in fetal lambs: a role for NADPH oxidase. *Circ Res* 92:683–691
- Buysse C, Fonteyne C, Dessy H, De Laet MH, Biarent D (2001) The use of dipyridamole to wean from inhaled nitric oxide in congenital diaphragmatic hernia. *J Pediatr Surg* 36(12):1864–1865
- Cai J, Su Z, Shi Z, Zhou Y, Xu Z, Liu J, Chen L, Xu Z, Yu X, Ding W, Yang Y (2008a) Nitric oxide in conjunction with milrinone better stabilized pulmonary hemodynamics after Fontan procedure. *Artif Organs* 32(11):864–869
- Cai J, Su Z, Shi Z, Zhou Y, Xu Z, Xu Z, Yang Y (2008b) Nitric oxide and milrinone: combined effect on pulmonary circulation after Fontan-type procedure: a prospective, randomized study. *Ann Thorac Surg* 86(3):882–888, discussion 882–888
- Chang AC, Atz AM, Wernovsky G, Burke RP, Wessel DL (1995) Milrinone: systemic and pulmonary hemodynamic effects in neonates after cardiac surgery. *Crit Care Med* 23(11):1907–1914
- Chen B, Lakshminrusimha S, Czech L, Groh BS, Gugino SF, Russell JA, Farrow KN, Steinhorn RH (2009) Regulation of phosphodiesterase 3 in the pulmonary arteries during the perinatal period in sheep. *Pediatr Res* 66(6):682–687
- Clark RH, Kueser TJ, Walker MW, Southgate WM, Huckaby JL, Perez JA, Roy BJ, Keszler M, Kinsella JP (2000) Low-dose nitric oxide therapy for persistent pulmonary hypertension of the newborn Clinical Inhaled Nitric Oxide Research Group. *N Engl J Med* 342(7):469–474
- Clark RH, Huckaby JL, Kueser TJ, Walker MW, Southgate WM, Perez JA, Roy BJ, Keszler M (2003) Low-dose nitric oxide therapy for persistent pulmonary hypertension: 1-year follow-up. *J Perinatol* 23(4):300–303
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- de Buys Roessingh AS, Dinh-Xuan AT (2009) Congenital diaphragmatic hernia: current status and review of the literature. *Eur J Pediatr* 168(4):393–406
- de Visser YP, Walther FJ, Laghmani EH, van Wijngaarden S, Nieuwland K, Wagenaar GT (2008) Phosphodiesterase-4 inhibition attenuates pulmonary inflammation in neonatal lung injury. *Eur Respir J* 31(3):633–644
- de Visser YP, Walther FJ, Laghmani el H, Boersma H, van der Laarse A, Wagenaar GT (2009) Sildenafil attenuates pulmonary inflammation and fibrin deposition, mortality and right ventricular hypertrophy in neonatal hyperoxic lung injury. *Respir Res* 10:30
- Dukarm RC, Morin FC 3rd, Russell JA, Steinhorn RH (1998) Pulmonary and systemic effects of the phosphodiesterase inhibitor dipyridamole in newborn lambs with persistent pulmonary hypertension. *Pediatr Res* 44(6):831–837
- Dukarm RC, Steinhorn RH, Russell JA, Lakshminrusimha S, Swartz D, Cummings JJ (2005) Selective type 5 phosphodiesterase inhibition alters pulmonary hemodynamics and lung liquid production in near term fetal lambs. *J Appl Physiol* 99(6):2331–2336

- Evgenov OV, Busch CJ, Evgenov NV, Liu R, Petersen B, Falkowski GE, Petho B, Vas A, Bloch KD, Zapol WM, Ichinose F (2006) Inhibition of phosphodiesterase 1 augments the pulmonary vasodilator response to inhaled nitric oxide in awake lambs with acute pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 290(4):L723–L729
- Fan Chung K (2006) Phosphodiesterase inhibitors in airways disease. *Eur J Pharmacol* 533 (1–3):110–117
- Farrow KN, Fliman P, Steinhorn RH (2005) The diseases treated with ECMO: focus on PPHN. *Semin Perinatol* 29(1):8–14
- Farrow KN, Groh BS, Schumacker PT, Lakshminrusimha S, Czech L, Gugino SF, Russell JA, Steinhorn RH (2008a) Hyperoxia increases phosphodiesterase 5 expression and activity in ovine fetal pulmonary artery smooth muscle cells. *Circ Res* 102(2):226–233
- Farrow KN, Lakshminrusimha S, Reda WJ, Wedgwood S, Czech L, Gugino SF, Davis JM, Russell JA, Steinhorn RH (2008b) Superoxide dismutase restores eNOS expression and function in resistance pulmonary arteries from neonatal lambs with persistent pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 295(6):L979–L987
- Farrow KN, Lakshminrusimha S, Czech L, Groh BS, Gugino SF, Davis JM, Russell JA, Steinhorn RH (2010a) Superoxide dismutase and inhaled nitric oxide normalize phosphodiesterase 5 expression and activity in neonatal lambs with persistent pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 299(1):L109–L116
- Farrow KN, Wedgwood S, Lee KJ, Czech L, Gugino SF, Lakshminrusimha S, Schumacker PT, Steinhorn RH (2010b) Mitochondrial oxidant stress increases PDE5 activity in persistent pulmonary hypertension of the newborn. *Respir Physiol Neurobiol*. doi:10.1016/j.resp.2010.08.018
- Galie N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzyna M, Simonneau G (2005) Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med* 353(20):2148–2157
- Gelfand SL, Fanaroff JM, Walsh MC (2004) Controversies in the treatment of meconium aspiration syndrome. *Clin Perinatol* 31(3):445–452
- Giaid A, Saleh D (1995) Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. *N Engl J Med* 333(4):214–221
- Group, N.I.N.O.S (1997) Inhaled nitric oxide in full-term and nearly full-term infants with hypoxic respiratory failure. *New Eng J Med* 336:597–604
- Group, N.I.N.O.S (2000) Inhaled nitric oxide in term and near-term infants: neurodevelopmental follow-up of the neonatal inhaled nitric oxide study group (NINOS). *J Pediatr* 136(5):611–617
- Hanson KA, Burns F, Rybalkin SD, Miller JW, Beavo J, Clarke WR (1998a) Developmental changes in lung cGMP phosphodiesterase-5 activity, protein, and message. *Am J Respir Crit Care Med* 158(1):279–288
- Hanson KA, Ziegler JW, Rybalkin SD, Miller JW, Abman SH, Clarke WR (1998b) Chronic pulmonary hypertension increases fetal lung cGMP phosphodiesterase activity. *Am J Physiol* 275(5 Pt 1):L931–L941
- Haworth SG (1988) Pulmonary vascular remodeling in neonatal pulmonary hypertension. *Chest* 93:133S–138S
- Hoffman JI, Rudolph AM, Heymann MA (1981) Pulmonary vascular disease with congenital heart lesions: pathologic features and causes. *Circulation* 64(5):873–877
- Hoffman TM, Wernovsky G, Atz AM, Kulik TJ, Nelson DP, Chang AC, Bailey JM, Akbary A, Kocsis JF, Kaczmarek R, Spray TL, Wessel DL (2003) Efficacy and safety of milrinone in preventing low cardiac output syndrome in infants and children after corrective surgery for congenital heart disease. *Circulation* 107(7):996–1002
- Houslay MD, Schafer P, Zhang KY (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10(22):1503–1519
- Ivy DD, Kinsella JP, Ziegler JW, Abman SH (1998) Dipyridamole attenuates rebound pulmonary hypertension after inhaled nitric oxide withdrawal in postoperative congenital heart disease. *J Thorac Cardiovasc Surg* 115(4):875–882

- Jobe AH, Bancalari E (2001) Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 163(7): 1723–1729
- Karamanoukian HL, Peay T, Love JE, Abdel-Rahman E, Dandonna P, Azizkhan RG, Glick PL (1996) Decreased pulmonary nitric oxide synthase activity in the rat model of congenital diaphragmatic hernia. *J Pediatr Surg* 31(8):1016–1019
- Keller RL, Hamrick SE, Kitterman JA, Fineman JR, Hawgood S (2004) Treatment of rebound and chronic pulmonary hypertension with oral sildenafil in an infant with congenital diaphragmatic hernia. *Pediatr Crit Care Med* 5(2):184–187
- Khemani E, McElhinney DB, Rhein L, Andrade O, Lacro RV, Thomas KC, Mullen MP (2007) Pulmonary artery hypertension in formerly premature infants with bronchopulmonary dysplasia: clinical features and outcomes in the surfactant era. *Pediatrics* 120(6):1260–1269
- Kinsella JP, Cutter GR, Walsh WF, Gerstmann DR, Bose CL, Hart C, Sekar KC, Auten RL, Bhutani VK, Gerdes JS, George TN, Southgate WM, Carriedo H, Couser RJ, Mammel MC, Hall DC, Pappagallo M, Sardesai S, Strain JD, Baier M, Abman SH (2006) Early inhaled nitric oxide therapy in premature newborns with respiratory failure. *N Engl J Med* 355(4):354–364
- Konduri GG (2004) New approaches for persistent pulmonary hypertension of newborn. *Clin Perinatol* 31(3):591–611
- Konduri GG, Ou J, Shi Y, Pritchard KA (2003) Decreased association of hsp90 impairs endothelial nitric oxide synthase in fetal lambs with persistent pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 285:H204–H211
- Ladha F, Bonnet S, Eaton F, Hashimoto K, Korbitt G, Thebaud B (2005) Sildenafil improves alveolar growth and pulmonary hypertension in hyperoxia-induced lung injury. *Am J Respir Crit Care Med* 172:750–756
- Lakshminrusimha S, Steinhorn RH (1999) Pulmonary vascular biology during neonatal transition. *Clin Perinatol* 26(3):601–619
- Lakshminrusimha S, Steinhorn RH (2009) Phosphodiesterase inhibitors in the management of persistent pulmonary hypertension of the newborn (PPHN). *eNeonatal Review* 6(12) <http://www.hopkinscme.edu/ofp/eNeonatalReview/Newsletters/2009/0909.html>
- Lakshminrusimha S, Russell JA, Steinhorn RH, Ryan RM, Gugino SF, Morin FC 3rd, Swartz DD, Kumar VH (2006) Pulmonary arterial contractility in neonatal lambs increases with 100% oxygen resuscitation. *Pediatr Res* 59(1):137–141
- Lakshminrusimha S, Russell JA, Steinhorn RH, Swartz DD, Ryan RM, Gugino SF, Wynn KA, Kumar VH, Mathew B, Kirmani K, Morin FC 3rd (2007a) Pulmonary hemodynamics in neonatal lambs resuscitated with 21%, 50%, and 100% oxygen. *Pediatr Res* 62(3):313–318
- Lakshminrusimha S, Wiseman D, Black SM, Russell JA, Gugino SF, Oishi P, Steinhorn RH, Fineman JR (2007b) The role of nitric oxide synthase-derived reactive oxygen species in the altered relaxation of pulmonary arteries from lambs with increased pulmonary blood flow. *Am J Physiol Heart Circ Physiol* 293(3):H1491–H1497
- Lakshminrusimha S, Porta NF, Farrow KN, Chen B, Gugino SF, Kumar VH, Russell JA, Steinhorn RH (2009a) Milrinone enhances relaxation to prostacyclin and iloprost in pulmonary arteries isolated from lambs with persistent pulmonary hypertension of the newborn. *Pediatr Crit Care Med* 10(1):106–112
- Lakshminrusimha S, Swartz DD, Gugino SF, Ma CX, Wynn KA, Ryan RM, Russell JA, Steinhorn RH (2009b) Oxygen concentration and pulmonary hemodynamics in newborn lambs with pulmonary hypertension. *Pediatr Res* 66(5):539–544
- Lee JE, Hillier SC, Knoderer CA (2008) Use of sildenafil to facilitate weaning from inhaled nitric oxide in children with pulmonary hypertension following surgery for congenital heart disease. *J Intensive Care Med* 23(5):329–334
- Leffler CW, Hessler JR, Green RS (1984) The onset of breathing at birth stimulates pulmonary vascular prostacyclin synthesis. *Pediatr Res* 18:938–942
- Levin DL, Rudolph AM, Heymann MA, Phibbs RH (1976) Morphological development of the pulmonary vascular bed in fetal lambs. *Circulation* 53:144–151

- Loughney K, Hill TR, Florio VA, Uher L, Rosman GJ, Wolda SL, Jones BA, Howard ML, McAllister-Lucas LM, Sonnenburg WK, Francis SH, Corbin JD, Beavo JA, Ferguson K (1998) Isolation and characterization of cDNA encoding PDE5A, a human cGMP-binding, cGMP-specific 3', 5'-cyclic nucleotide phosphodiesterase. *Gene* 216:139–147
- Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 109(3):366–398
- Maclean MR, Johnston ED, McCulloch KM, Pooley L, Houslay MD, Sweeney G (1997) Phosphodiesterase isoforms in the pulmonary arterial circulation of the rat: changes in pulmonary hypertension. *J Pharmacol Exp Ther* 283(2):619–624
- Manchester D, Margolis HS, Sheldon RE (1976) Possible association between maternal indomethacin therapy and primary pulmonary hypertension of the newborn. *Am J Obstet Gynecol* 126:467–469
- McNamara PJ, Laique F, Muang-In S, Whyte HE (2006) Milrinone improves oxygenation in neonates with severe persistent pulmonary hypertension of the newborn. *J Crit Care* 21(2):217–222
- Millen J, MacLean MR, Houslay MD (2006) Hypoxia-induced remodelling of PDE4 isoform expression and cAMP handling in human pulmonary artery smooth muscle cells. *Eur J Cell Biol* 85(7):679–691
- Morin FC 3rd (1989) Ligating the ductus arteriosus before birth causes persistent pulmonary hypertension in the newborn lamb. *Pediatr Res* 25:245–250
- Mourani PM, Sontag MK, Younoszai A, Ivy DD, Abman SH (2008) Clinical utility of echocardiography for the diagnosis and management of pulmonary vascular disease in young children with chronic lung disease. *Pediatrics* 121(2):317–325
- Mourani PM, Sontag MK, Ivy DD, Abman SH (2009) Effects of long-term sildenafil treatment for pulmonary hypertension in infants with chronic lung disease. *J Pediatr* 154(3):379–384, 384 e1–e2
- Mukherjee A, Dombi T, Wittke B, Lalonde R (2009) Population pharmacokinetics of sildenafil in term neonates: evidence of rapid maturation of metabolic clearance in the early postnatal period. *Clin Pharmacol Ther* 85(1):56–63
- Murphy JD, Rabinovitch M, Goldstein JD, Reid LM (1981) The structural basis of persistent pulmonary hypertension of the newborn infant. *J Pediatr* 98(6):962–967
- Murray F, Patel HH, Suda RY, Zhang S, Thistlethwaite PA, Yuan JX, Insel PA (2007) Expression and activity of cAMP phosphodiesterase isoforms in pulmonary artery smooth muscle cells from patients with pulmonary hypertension: role for PDE1. *Am J Physiol Lung Cell Mol Physiol* 292(1):L294–L303
- Namachivayam P, Theilen U, Butt WW, Cooper SM, Penny DJ, Shekerdemian LS (2006) Sildenafil prevents rebound pulmonary hypertension after withdrawal of nitric oxide in children. *Am J Respir Crit Care Med* 174(9):1042–1047
- Noori S, Friedlich P, Wong P, Garingo A, Seri I (2007) Cardiovascular effects of sildenafil in neonates and infants with congenital diaphragmatic hernia and pulmonary hypertension. *Neonatology* 91(2):92–100
- Okogbule-Wonodi AC, Ibe BO, Yue BW, Hsu S, Raj JU (1998) Phosphodiesterase activity in intrapulmonary arteries and veins of perinatal lambs. *Mol Genet Metab* 65(3):229–237
- Rabinovitch M, Haworth SG, Castaneda AR, Nadas AS, Reid LM (1978) Lung biopsy in congenital heart disease: a morphometric approach to pulmonary vascular disease. *Circulation* 58(6):1107–1122
- Rabinovitch M, Keane JF, Norwood WI, Castaneda AR, Reid L (1984) Vascular structure in lung tissue obtained at biopsy correlated with pulmonary hemodynamic findings after repair of congenital heart defects. *Circulation* 69(4):655–667
- Rabinovitch M, Bothwell T, Hayakawa BN, Williams WG, Trusler GA, Rowe RD, Olley PM, Cutz E (1986) Pulmonary artery endothelial abnormalities in patients with congenital heart defects and pulmonary hypertension. A correlation of light with scanning electron microscopy and transmission electron microscopy. *Lab Invest* 55(6):632–653

- Reddy VM, Wong J, Liddicoat JR, Johengen M, Chang R, Fineman JR (1996) Altered endothelium-dependent responses in lambs with pulmonary hypertension and increased pulmonary blood flow. *Am J Physiol* 271(2 Pt 2):H562–H570
- Ross GA, Oishi P, Azakie A, Fratz S, Fitzgerald RK, Johengen MJ, Harmon C, Hendricks-Munoz K, Xu J, Black SM, Fineman JR (2005) Endothelial alterations during inhaled NO in lambs with pulmonary hypertension: implications for rebound hypertension. *Am J Physiol Lung Cell Mol Physiol* 288(1):L27–L35
- Sanchez LS, de la Monte SM, Filippov G, Jones RC, Zapol WM, Bloch KD (1998) Cyclic-GMP-binding, cyclic-GMP-specific phosphodiesterase (PDE5) gene expression is regulated during rat pulmonary development. *Pediatr Res* 43(2):163–168
- Schermuly RT, Pullamsetti SS, Kwapiszewska G, Dumitrascu R, Tian X, Weissmann N, Ghofrani HA, Kaulen C, Dunkern T, Schudt C, Voswinckel R, Zhou J, Samidurai A, Klepetko W, Paddenberg R, Kummer W, Seeger W, Grimminger F (2007) Phosphodiesterase 1 upregulation in pulmonary arterial hypertension: target for reverse-remodeling therapy. *Circulation* 115(17):2331–2339
- Shah PS, Ohlsson A (2007) Sildenafil for pulmonary hypertension in neonates. *Cochrane Database Syst Rev* 3:CD005494
- Shaul PW, Wells LB (1994) Oxygen modulates nitric oxide production selectively in fetal pulmonary endothelial cells. *Am J Respir Crit Care Med* 11:432–438
- Shaul PW, Campbell WB, Farrar MA, Magness RR (1992) Oxygen modulates prostacyclin synthesis in ovine fetal pulmonary arteries by an effect on cyclooxygenase. *J Clin Invest* 90:2147–2155
- Shaul PW, Yuhanna IS, German Z, Chen Z, Steinhorn RH, Morin FC 3rd (1997) Pulmonary endothelial NO synthase gene expression is decreased in fetal lambs with pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 272:L1005–L1012
- Shaul PW, Afshar S, Gibson LL, Sherman TS, Kerecman JD, Grubb PH, Yoder BA, McCurnin DC (2002) Developmental changes in nitric oxide synthase isoform expression and nitric oxide production in fetal baboon lung. *Am J Physiol Lung Cell Mol Physiol* 283(6):L1192–L1199
- Shekerdemian LS, Ravn HB, Penny DJ (2002) Intravenous sildenafil lowers pulmonary vascular resistance in a model of neonatal pulmonary hypertension. *Am J Respir Crit Care Med* 165(8):1098–1102
- Shekerdemian LS, Ravn HB, Penny DJ (2004) Interaction between inhaled nitric oxide and intravenous sildenafil in a porcine model of meconium aspiration syndrome. *Pediatr Res* 55(3):413–418
- Steinhorn RH, Morin FC 3rd, Van Wylen DG, Gugino SF, Giese EC, Russell JA (1994) Endothelium-dependent relaxations to adenosine in juvenile rabbit pulmonary arteries and veins. *Am J Physiol* 266:H2001–H2006
- Steinhorn RH, Russell JA, Morin FC 3rd (1995) Disruption of cGMP production in pulmonary arteries isolated from fetal lambs with pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 268:H1483–H1489
- Steinhorn RH, Russell JA, Lakshminrusimha S, Gugino SF, Black SM, Fineman JR (2001) Altered endothelium-dependent relaxations in lambs with high pulmonary blood flow and pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 280(1):H311–H317
- Steinhorn RH, Kinsella JP, Pierce C, Butrous G, Dilleen M, Oakes M, Wessel DL (2009) Intravenous sildenafil in the treatment of neonates with persistent pulmonary hypertension. *J Pediatr* 155(6):841–847 e1
- Stocker C, Penny DJ, Brizard CP, Cochrane AD, Soto R, Shekerdemian LS (2003) Intravenous sildenafil and inhaled nitric oxide: a randomised trial in infants after cardiac surgery. *Intensive Care Med* 29(11):1996–2003
- Thebaud B, Saizou C, Farnoux C, Hartman JF, Mercier JC (1999) Dypiridamole, a cGMP phosphodiesterase inhibitor, transiently improves the response to inhaled nitric oxide in two newborns with congenital diaphragmatic hernia. *Intensive Care Med* 25(3):300–303

- Thebaud B, Ladha F, Michelakis ED, Sawicka M, Thurston G, Eaton F, Hashimoto K, Harry G, Haromy A, Korbitt G, Archer SL (2005) Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. *Circulation* 112(16):2477–2486
- Thelitz S, Oishi P, Sanchez LS, Bekker JM, Ovidia B, Johengen MJ, Black SM, Fineman JR (2004) Phosphodiesterase-3 inhibition prevents the increase in pulmonary vascular resistance following inhaled nitric oxide withdrawal in lambs. *Pediatr Crit Care Med* 5(3):234–239
- Thusu KG, Morin FC 3rd, Russell JA, Steinhorn RH (1995) The cGMP phosphodiesterase inhibitor zaprinast enhances the effect of nitric oxide. *Am J Respir Crit Care Med* 152(5 Pt 1): 1605–1610
- Tiktinsky MH, Morin FC 3rd (1993) Increasing oxygen tension dilates fetal pulmonary circulation via endothelium-derived relaxing factor. *Am J Physiol* 265(1 Pt 2):H376–H380
- Tzao C, Nickerson PA, Russell JA, Gugino SF, Steinhorn RH (2001) Pulmonary hypertension alters soluble guanylate cyclase activity and expression in pulmonary arteries isolated from fetal lambs. *Pediatr Pulmonol* 31(2):97–105
- Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, Nerem RM, Harrison DG (1995) Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am J Physiol* 269:C1371–C1378
- Vento M, Asensi M, Sastre J, Garcia-Sala F, Pallardo FV, Vina J (2001) Resuscitation with room air instead of 100% oxygen prevents oxidative stress in moderately asphyxiated term neonates. *Pediatrics* 107(4):642–647
- Villanueva ME, Zaher FM, Svinarich DM, Konduri GG (1998) Decreased gene expression of endothelial nitric oxide synthase in newborns with persistent pulmonary hypertension. *Pediatr Res* 44:338–343
- Vukcevic Z, Coppola CP, Hults C, Gosche JR (2005) Nitrovasodilator responses in pulmonary arterioles from rats with nitrofen-induced congenital diaphragmatic hernia. *J Pediatr Surg* 40(11): 1706–1711
- Walsh MC, Szeffler S, Davis J, Allen M, Van Marter L, Abman S, Blackmon L, Jobe A (2006) Summary proceedings from the bronchopulmonary dysplasia group. *Pediatrics* 117(3 Pt 2): S52–S56
- Walsh-Sukys MC, Tyson JE, Wright LL, Bauer CR, Korones SB, Stevenson DK, Verter J, Stoll BJ, Lemons JA, Papile L, Shankaran S, Donovan EF, Oh W, Ehrenkranz RA, Fanaroff AA (2000) Persistent pulmonary hypertension of the newborn in the era before nitric oxide: practice variation and outcomes. *Pediatrics* 105:14–20
- Wild LM, Nickerson PA, Morin FC 3rd (1989) Ligating the ductus arteriosus before birth remodels the pulmonary vasculature of the lamb. *Pediatr Res* 25:251–257
- Worwag S, Mulla H, Luyt D, Firmin RK (2000) Dipyridamole in the treatment of a neonate with persistent pulmonary hypertension. *J R Soc Med* 93(2):77–78
- Woyda K, Koeblich S, Reiss I, Rudloff S, Pullamsetti SS, Ruhlmann A, Weissmann N, Ghofrani HA, Gunther A, Seeger W, Grimminger F, Morty RE, Schermuly RT (2009) Inhibition of phosphodiesterase 4 enhances lung alveolarisation in neonatal mice exposed to hyperoxia. *Eur Respir J* 33(4):861–870
- Zaccolo M, Movsesian MA (2007) cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res* 100(11):1569–1578

Role of Phosphodiesterases in Adult-Onset Pulmonary Arterial Hypertension

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Abstract Pulmonary arterial hypertension (PAH) is characterized by increased mean pulmonary artery pressure (mPAP) due to vasoconstriction and structural changes in the small pulmonary arteries (PAs); proliferation of pulmonary artery smooth muscle cells (PASMCs) contributes to the remodeling. The abnormal pathophysiology in the pulmonary vasculature relates to decreased cyclic nucleotide levels in PASMCs. Phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP and cGMP, thereby PDE inhibitors are effective in vasodilating the PA and decreasing PASMC proliferation. Experimental studies support the use of PDE3, PDE5, and PDE1 inhibitors in PAH. PDE5 inhibitors such as sildenafil are clinically approved to treat different forms of PAH and lower mPAP, increase functional capacity, and decrease right ventricular hypertrophy, without decreasing systemic arterial pressure. New evidence suggests that the combination of PDE inhibitors with other therapies

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for PAH may be beneficial in treating the disease. Furthermore, inhibiting PDEs in the heart and the inflammatory cells that infiltrate the PA may offer new targets to reduce right ventricular hypertrophy and inhibit inflammation that is associated with and contributes to the development of PAH. This chapter summarizes the advances in the area and the future of PDEs in PAH.

Keywords Inflammation · Proliferation · Pulmonary arterial hypertension · Pulmonary artery · Pulmonary artery smooth muscle cells · Right ventricular hypertrophy · Vasodilation

1 Pulmonary Arterial Hypertension

The normal pulmonary circulation is a low pressure (mean pulmonary artery pressure [mPAP] of ~15 mmHg), low resistance (1/8 of the systemic), high flow circulation, which unlike the systemic circulation is maximally dilated at all times. The highly compliant structure of the pulmonary arterial tree and the circulating and locally produced vasoactive mediators that regulate pulmonary vascular tone and permeability maintain the low-pressure environment and allow the pulmonary vasculature to accommodate the entire cardiac output (Barnes and Liu 1995; Humbert et al. 2004; Morrell et al. 2009; Tuder et al. 2009). An imbalance of vasoconstrictor/co-mitogen and vasodilator/antiproliferative mediators in the pulmonary circulation leads to vasoconstriction, cellular proliferation, and thrombosis, and thereby increased pulmonary vascular resistance (PVR), which can manifest as pulmonary hypertension (PH). The World Health Organization (WHO) has classified PH into five major groups, which include Group 1, pulmonary arterial hypertension (PAH), Group 2, PH with left heart disease, Group 3, PH associated with lung diseases and/or hypoxemia, Group 4, PH due to chronic thrombotic and/or embolic disease, and Group 5, miscellaneous causes of PH. Group 1, termed PAH, which is the focus of this chapter, is further classified and comprises patients with similar pathophysiological, histological, and prognostic features (Table 1) (Galie et al. 2009c; McLaughlin et al. 2009; Simonneau et al. 2009). PAH is a disease that is associated with multiple genetic, molecular, and humoral abnormalities that can lead to right ventricular failure and death.

PAH is defined as a resting mPAP >25 mmHg or >30 mmHg with exercise, pulmonary arterial wedge pressure of \leq 15 mmHg and increased PVR. The prevalence of PAH is around 15 per million (although this varies depending on the underlying condition and between national databases) and affects people of all races and cultures, usually developing in adults between the ages of 20 and 60 (mean ~ age 48), (Humbert et al. 2006; Peacock et al. 2007). PAH in adults can be idiopathic (IPAH, incidence of 2.4 per million), heritable (formally called familial), or secondary to drug/toxin exposure or to other conditions such as congenital heart disease, connective tissue disorder, portal hypertension or HIV. Table 1 outlines the updated clinical classification for PAH (Galie et al. 2009b; McLaughlin et al. 2009;

Table 1 Outline of the updated clinical classification of pulmonary arterial hypertension (PAH), modified from (Galie et al. 2009c; McLaughlin et al. 2009; Simonneau et al. 2009)

Clinical classification of pulmonary arterial hypertension (Group 1)
1.1 Idiopathic
1.2 Heritable
1.2.1 BMPR2
1.2.2 ALK-1, endoglin
1.2.3 Unknown
1.3 Induced by drugs and toxins
1.4 Associated with:
1.4.1 Connective tissue disease
1.4.2 HIV
1.4.3 Portal hypertension
1.4.4 Congenital heart disease
1.4.5 Schistosomiasis
1.4.6 Chronic hemolytic anemia
1.5 Persistent pulmonary hypertension of the newborn

Simonneau et al. 2009). Younger women are more than twice as likely to develop IPAH (life expectancy <3 years if untreated), which has an unknown etiology; therefore, one focus of current research is to determine the link between gender and PAH (D’Alonzo et al. 1991; Humbert et al. 2006; Peacock et al. 2007).

Heritable PAH, in around 70% of cases, has been shown to be the result of mutations in the bone morphogenetic protein receptor 2 (BMPR2) gene; more recently, mutations in receptor members of the transforming growth factor-β (TGF-β), activin-like kinase-type 1 and endoglin families, have been implicated in a subset of patients (Chaouat et al. 2004; Lane et al. 2000; Machado et al. 2001; Trembath 2001). Mutations in BMPR2 include frameshifts, partial deletions, insertions, missense, and splice-site mutations, and although many reduce BMPR2 expression and lead to dysfunctional SMAD signaling and an increase in TGF-β signaling, the full impact of these mutations still needs to be determined (Machado et al. 2009). Interestingly, animal models of PAH are also associated with a reduction in the expression of BMPR2 mRNA, protein and BMP signaling, suggesting that the alteration in BMP/TGF-β pathways may also underlie other forms of PAH (Long et al. 2009).

Despite different mechanisms of PAH, much of the remodeling in the lung is similar and the prognosis is poor for all forms; therefore, novel therapeutic approaches are much needed. Current treatments for PAH (Group 1) include anti-coagulants, diuretics, digoxin, supplemental oxygen, calcium-channel blockers, prostanoids, endothelin-receptor antagonists, and phosphodiesterase-5 inhibitors, the focus of this chapter (Galie et al. 2009b; McLaughlin et al. 2009; Simonneau et al. 2009). Future successful treatments of PAH will likely require a better understanding of the physiology, pathophysiology, and triggers of the disease.

The adventitial, medial, and intimal thickening that leads to the remodeling of the pulmonary artery (PA) and the development of PAH are the result of the complex interaction between activation of fibroblasts and smooth muscle cells, endothelial dysfunction, and infiltration of inflammatory mediators such as

monocytes/macrophages, T- and B-lymphocytes, and dendritic cells (Barnes and Liu 1995; Humbert et al. 2004; Morrell et al. 2009; Tuder et al. 2009). Although each cell type contributes to PAH, increased vasoconstriction is currently the main target for investigative and approved therapies. Studies of PAH often involve use of animal models, most commonly, the chronically hypoxic rat/mouse and monocrotaline-treated rat (Stenmark et al. 2009). Genetically manipulated mice are used to study the role of specific genes in the pathogenesis of PAH: mice overexpressing the serotonin transporter develop elevated mPAP and are more susceptible to hypoxia-induced PAH (Dempsey et al. 2008). Although such models have provided insight into mediators of the disease and aided in the development of drugs for PAH, species-specific function of certain targets, which will be discussed below in relation to PDE1, may have resulted in their importance being overlooked.

2 Role of Cyclic Nucleotides in PAH

Both adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) are intracellular second messengers that vasodilate pulmonary vessels. Increased levels of cyclic nucleotides lower $[Ca^{2+}]_i$ to relax PAs, decrease pulmonary artery smooth muscle cells (PASMCs) proliferation, and increase PASMC apoptosis (Koyama et al. 2001; Rybalkin and Bornfeldt 1999). In PASMCs, cGMP exerts the majority of its actions through activation of protein kinase G (PKG), whereas cAMP actions are mediated via protein kinase A (PKA) and Epac [Exchange protein directly activated by cAMP; Epac-1 and Epac-2 are known (Grandoch et al. 2010)]; the relative importance of these mediators in the vasodilation of the pulmonary circulation is currently under investigation. The PAs from rats with experimental PAH and PASMCs isolated from PAH patients have decreased cyclic nucleotide levels (MacLean et al. 1996, 1997; Murray et al. 2007). Many endogenous pulmonary vasodilators and therapies for PAH, such as nitric oxide (NO) and stable analogues for prostacyclin, act by increasing the cellular content of cGMP and cAMP, respectively. Increasing $[cGMP]_i$, by addition of NO or natriuretic peptides (atrial natriuretic peptide [ANP] and brain natriuretic peptide [BNP]), reduces mPAP and PVR in experimental models of PAH; however, since they require continuous delivery, such agents are difficult to use clinically (Baliga et al. 2008; Ghofrani et al. 2002). Epoprostenol, treprostinil, and iloprost, which are stable prostacyclin analogues, are approved to treat PAH; in severe PAH, iloprost is effective in ~15% of patients (Ghofrani et al. 2002; Olschewski et al. 2002). The therapeutic potential of prostacyclin analogues is hampered somewhat by issues related to their delivery and the development of tolerance, hence new drugs that act via increasing cyclic nucleotides are required.

3 Targeting Phosphodiesterases in PAH

The rationale for targeting cyclic nucleotide phosphodiesterases (PDEs) in PAH is based on the favorable effects achieved by increasing the intracellular concentration of cAMP and cGMP, and the idea that the amplitude and duration of their increase in concentration can be enhanced by decreasing their degradation. Tissue-related variations in distribution and physiological function of PDEs make them good pharmacological targets for PAH, i.e., by tailoring cyclic nucleotide signaling in a pulmonary-specific manner (Bender and Beavo 2006). For example, since PDE5 and other components of the cGMP-signaling pathway are abundant in the lung, this helps explain the interest and success of PDE5 inhibitors in effectively promoting pulmonary vascular smooth muscle relaxation (Corbin et al. 2005). Activities of both cAMP-PDE and cGMP-PDE are increased in the PA from experimental models of PAH, which is also true in PASMCS isolated from PAH patients (Maclean et al. 1997; Murray et al. 2007; Wharton et al. 2005). The increased PDE activity has important consequences: unless a PDE inhibitor is present, cAMP accumulation in PAH-PASMCS in response to agonists that increase cAMP synthesis, for example, forskolin or beraprost (an analogue of prostacyclin) is attenuated compared to that of control-PASMCS (Murray et al. 2007).

In the lung, the main PDEs that control cyclic nucleotide levels were initially shown to be PDE3 (cGMP-inhibited, cAMP-specific) and PDE5 (cGMP-specific); inhibitors for each of these PDEs relax isolated precontracted human and rat PAs (Cohen et al. 1996; Dent et al. 1994; Maclean et al. 1997; Phillips et al. 2005; Pyne et al. 2007; Rabe et al. 1994). Real-time polymerase chain reaction (PCR) data (Fig. 1a), although confirming that PDE3A and PDE5A are the highest expressed PDE isoforms in PASMCS, also detect mRNA expression of PDE1A, 1C, 2A, 3B, 4A, 4B, 4C, 4D, 7A, 7B, 8A, 9A, 9B, 10A, and 11A, thereby suggesting that a number of other PDEs could contribute to the maintenance of low pulmonary vascular tone. In all patients with PAH-PASMCS whom we studied, we found increased expression of PDE1A, PDE1C, PDE3B, PDE5A, and PDE7A (PDE3A was only increased in IPAH patients, Fig. 1b): the increase in expression correlated with an increase in the relative contribution of PDE1 and PDE3 to total cAMP-PDE activity in PAH-PASMCS compared to control (Fig. 1c). PDE activity was determined using 1 μ M cAMP as substrate through a two-step radioassay procedure (Keravis et al. 2005; Thompson et al. 1974). Substrate and protein were incubated over a period of time that PDE activity was linear. To identify the contribution of specific PDEs, activity assays were performed in the presence of specific PDE inhibitors [PDE1, 30 μ M vinpocetine, 30 μ M 8-methoxy-methyl-3-isobutyl-1-methylxanthine (8-MM-IBMX); PDE2, 10 μ M erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) in the presence of excess cGMP; PDE3, 10 μ M milrinone; and PDE4, 10 μ M rolipram] and with or without Ca^{2+} /calmodulin in the presence of EGTA. In addition to being therapeutic targets for PAH, the upregulation of these PDE isoforms may provide insights into the mechanisms of the disease (Murray et al. 2007). In this chapter, we review the basic research and clinical data that

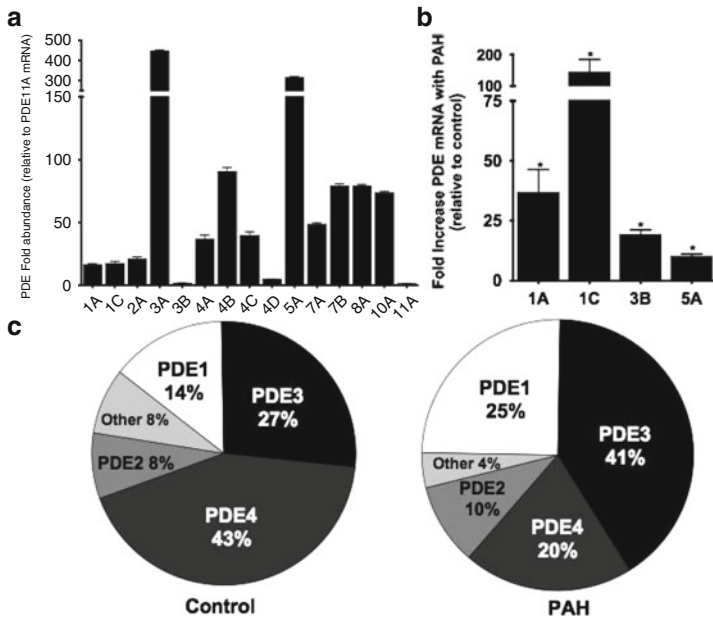


Fig. 1 Multiple PDE isoforms are expressed in PASMCs and upregulated with PAH. (a) Real-time PCR shows that mRNA of multiple PDEs are expressed in PASMCs, Ct normalized to PDE11. (b) PDE1A, 1C, 3B, 5A, and 7A are upregulated in PAH-PASMCs vs. control-PASMCs. (c) The relative contribution of PDE1 and PDE3 to total cAMP-PDE activity is increased with PAH. PDE activity was determined using 1 μ M cAMP as substrate through a two-step radioassay procedure (Keravis et al. 2005; Thompson et al. 1974). To identify the contribution of activity of specific PDEs, assays were performed in the presence of specific PDE inhibitors [PDE1, 30 μ M vinpocetine, 30 μ M 8-methoxy-methyl-3-isobutyl-1-methylxanthine (8-MM-IBMX); PDE2, 10 μ M erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) in the presence of excess cGMP; PDE3, 10 μ M milrinone; and PDE4, 10 μ M rolipram] and with or without Ca^{2+} /calmodulin in the presence of EGTA. PASMCs used in these experiments were between passage 4 and 6. $n = 5$, $*P < 0.05$ vs. control

support the role of PDEs in their vasodilator capacity in PAH and other aspects of the disease for which targeting of PDEs may be beneficial.

3.1 Targeting PDEs in Pulmonary Artery Vasoconstriction and Remodeling

3.1.1 Role of PDE5 in Pulmonary Vasculature

PDE5, the main cGMP-PDE in the lung, is thought to contribute to ~80% of cGMP hydrolysis in PASMCs and therefore is important for the regulation of normal pulmonary vascular tone (Ahn et al. 1991; Corbin et al. 2005; Fink et al. 1999;

Wharton et al. 2005). The level of expression of PDE5 is much higher in the pulmonary circulation than the systemic circulation, suggesting that pulmonary specificity is possible by targeting PDE5 (Corbin et al. 2005). With respect to PAH, increased expression, both at the level of mRNA and protein, increased phosphorylation and activity of PDE5 have been reported in a number of animal models of PAH, and importantly, also in patients with PAH, which would, at least in part, explain the disease-related decrease in cGMP levels and impaired endothelium-dependent pulmonary vasodilation (Black et al. 2001; Cohen et al. 1996; Hanson et al. 1998; Maclean et al. 1997; Murray et al. 2007; Wharton et al. 2005). In animal models of PAH, the PDE5 inhibitors zaprinast, E4021, and E4010, via increasing [cGMP]_I, vasodilate the pulmonary circulation; importantly, this vasodilation occurs without systemic effects (Cohen et al. 1996; Eddahibi et al. 1998; Hanasato et al. 1999; Ichinose et al. 1998; Jeffery and Wanstall 1998). E4010 was shown to improve mortality in monocrotaline-treated rats by 84% and in parallel to attenuate the development of hypoxia-induced PAH by reducing the increase in mPAP and the characteristic increase in medial wall thickness (Hanasato et al. 1999; Kodama and Adachi 1999). These data, together with those in Fig. 2a, which show that zaprinast significantly decreases serum-induced proliferation of PAH-PASMCs, suggest that in addition to being potent vasodilators PDE5 inhibitors are also antiproliferative and proapoptotic. However, since 10 μ M zaprinast can also block PDE1 activity, its effects could also be attributed to inhibition of this isoform.

The approval by the FDA of three oral PDE5 inhibitors for the treatment of erectile dysfunction, namely sildenafil (ViagraTM), tadalafil (CialisTM), and vardenafil (LevitraTM) opened up the possibility that PDE5 inhibitors could be used clinically to treat PAH. Evidence supports both the acute and the long-term use of these PDE5 inhibitors in a number of forms of experimental and clinical PAH. In 2007, the same formulation of sildenafil (marketed as Revatio) was approved for treatment of PAH, and the two other PDE5 inhibitors, tadalafil (marketed as Adcirca) and vardenafil (marketed as Levitra, used off-label), have more recently been tested in patients with PAH. Zhao et al. (2001) examined the effect of sildenafil on hypoxia-induced mice and healthy human volunteers in whom PAH was induced by inhaling a low oxygen concentration, which produced a 56% increase in mPAP (Zhao et al. 2001). A single dose of sildenafil (100 mg) inhibited the hypoxic rise in mPAP without systemic vasodilation and attenuated the increase in mPAP, RV hypertrophy, and remodeling in mice chronically exposed to hypoxia. Figure 2c, d show that sildenafil relaxes both the main and the first branch PA from the chronic-hypoxic rat, a response that is comparable to that in control-PA. It is important to highlight, which will be discussed below in further detail, that some of the effects of sildenafil may be due to partial blockade of PDE1 activity [since the IC₅₀ of sildenafil for PDE1 is achievable both in vivo and in vitro, (Paul et al. 2005)].

Clinically, sildenafil has been shown to be therapeutically useful in 80–90% of IPAH, and PAH secondary to HIV, thromboembolic pulmonary hypertension or portopulmonary hypertension: however, a lack of evidence has as-yet been

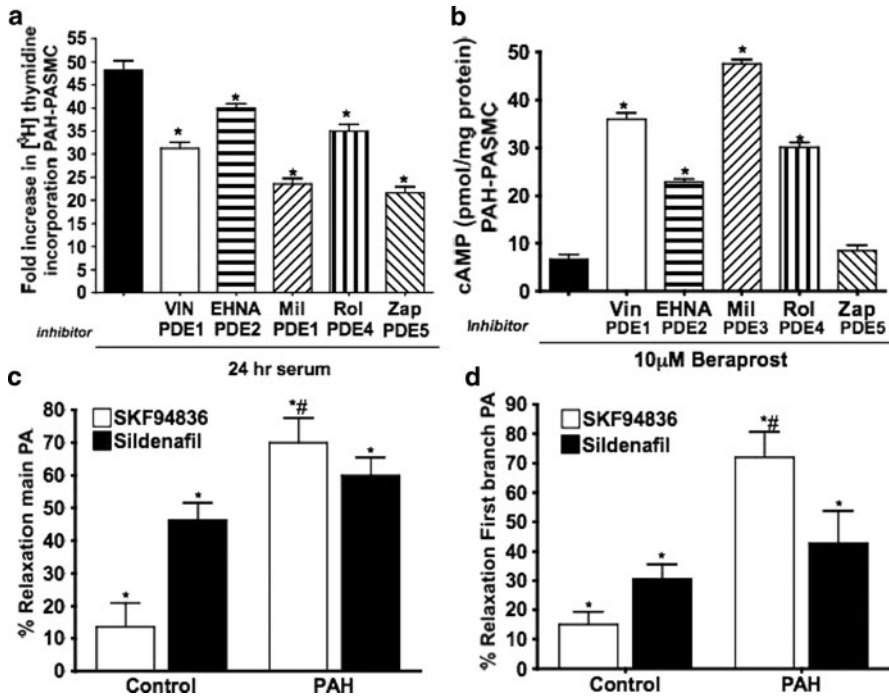


Fig. 2 PDE inhibitors decrease proliferation and increase cAMP in PAH-PASMCs and relax PA. (a) PDE inhibition [PDE1, vinpocetine (Vin); PDE2, erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA); PDE3, milrinone (Mil); PDE4, rolipram (Rol) and PDE5, zaprinast (Zap), all 10 μM] decreased serum-induced proliferation of PAH-PASMCs. (b) PDE inhibition increased cAMP accumulation, with the exception of PDE5 inhibition, in response to Beraprost (10 μM , 10 min). (c) Main and (d) first branch PA from control and chronic-hypoxic rat were contracted with phenylephrine (1 μM) and relaxation was measured after PDE3 (10 μM , SKF94836) and PDE5 inhibition (10 μM , Sildenafil). An organ bath preparation with an isometric force transducer was used to measure vessel tension in isolated PA. The concentration of PE used resulted in a sustained contraction that was approximately 80–90% of the maximum KCl response. $n = 5$ * $P < 0.05$ vs. minus PDE inhibitor # $P < 0.05$ vs. control PA

provided to show such benefit in other classes of pulmonary hypertension or that life expectancy is improved (Ghofrani et al. 2002; Michelakis et al. 2002; Wilkens et al. 2001). The first large-scale clinical trial to show the beneficial effects of sildenafil (20, 40, and 80 mg three times a day) for PAH was termed SUPER (Sildenafil Use for Pulmonary Hypertension, a study in which 30% of the patients had PAH). SUPER demonstrated that 80 mg sildenafil decreased PVR by 177–365 (mean 261) dyne s cm^{-5} , decreased PAP by 2.8–6.7 (mean 4.7) mmHg, and increased the 6-min walking distance by an average of 48 m over 12 weeks, an effect that was sustained for >1 year (Galie et al. 2005). Although sildenafil produced a dose-dependent decrease in PVR in PAH patients, the increase in the 6-min walk test was not, such that the 20 mg dose had an effect similar to that of both 40 and 80 mg. Explanation for the lack of dose–response of sildenafil may be

the result of changes in the affinity of sildenafil for PDE5 or in the conformational state of PDE5 on inhibitor binding (Francis et al. 1998; Mullershausen et al. 2003; Rybalkina et al. 2010). The FDA has approved the use of 20 mg sildenafil three times a day for PAH, although the dose can be increased to 40–80 mg or until side effects such as headache or dyspepsia develop.

Subsequently, the PHIRST (Pulmonary Arterial Hypertension and Response to Tadalafil) trial was undertaken, which demonstrated that vardenafil given 40 mg once per day (a single dose PDE5 inhibitor is thought to improve patient compliance) to PAH-patients for 16 weeks increased the 6-min walking distance by an average of 33 m, decreased mPAP and PVR and improved the time to clinical worsening, which led to FDA approval in June 2009 (Galie et al. 2009a). Vardenafil (5 mg once daily for the first 4 weeks, then 5 mg twice daily for 1 year) was tested in a multicenter, open-label study in PAH patients; this study showed comparable improvements in hemodynamic parameters and exercise capacity as was noted with sildenafil and tadalafil (Jing et al. 2009).

No clinical trials have directly compared the long-term therapeutic effects of all three of these inhibitors; however, in patients with IPAH, the acute effects of sildenafil, tadalafil, and vardenafil vary in terms of kinetics of pulmonary vasodilation (most rapid effect was vardenafil), pulmonary vascular selectivity (vardenafil also decreased systemic resistance), and ability to improve arterial oxygenation (only sildenafil improved oxygenation), strongly suggesting that longer-term studies are needed to determine whether such acute effects translate into clinical effectiveness (Ghofrani et al. 2004). Experimentally, although all three drugs dose-dependently relax isolated rat PAs, some data suggest that downstream signaling may differ among the PDE5 inhibitors. For example, only vardenafil markedly reduces CaCl_2 -induced contractions in phenylephrine-treated isolated PA, whereas only vardenafil inhibits the hypoxia-induced upregulation of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ in PA (Toque et al. 2008; Tsai et al. 2006).

As has been the case for the treatment of systemic hypertension, it is likely that combination therapy may be more efficacious in reducing mPAP and PA remodeling in PAH. A number of experimental and clinical studies have demonstrated that PDE5 inhibition additively or even synergistically improves pulmonary hemodynamics when used with a number of approved or experimental drugs for PAH. The combination of sildenafil with prostanoids, such as epoprostenol or inhaled iloprost, has been shown to be effective both experimentally and clinically and to decrease mPAP more than each agent alone (Ghofrani et al. 2003; Wilkens et al. 2001). In PAH patients, sildenafil reversed the decline in the 6-min walk test that was observed after three months of inhaled iloprost and sustained this benefit for over 1 year. Similarly, when the clinical benefits of an endothelin-A receptor antagonist declined in PAH patients, sildenafil increased the 6-min walk test by 43% and, importantly, no dose adjustment of either agent was required upon coadministration (Hoepfer et al. 2004; Stavros et al. 2010). In the chronic-hypoxic rat and when administered acutely to PAH patients, the decrease in mPAP and PVR by sildenafil can be augmented by the addition of ANP, BNP, or inhibitors of neutral endopeptidase; such data support the hypothesis that the beneficial effects

of sildenafil on pulmonary hemodynamics is dependent, in part, on the bioactivity of natriuretic peptides, the latter of which may be possible new therapeutic approaches (Baliga et al. 2008; Klinger et al. 2006). Thus, although long-term studies are required, therapy with PDE5 inhibitors appears safe and shows clinical benefit.

PDE5 inhibitors are known to enhance cGMP/PKG signaling but the mechanisms and downstream mediators for their antiproliferative and vasodilatory actions have not been fully elucidated; inhibition of PKG reverses many of the effects of sildenafil (Li et al. 2009; Sun et al. 2009b). PKG activation in PSMCs, via inhibition of PDE5, activates calcium pumps, inhibits voltage-gated and receptor operated Ca^{2+} channels and inhibits BK_{Ca} channels, all of which contribute to reduction in intracellular $[\text{Ca}^{2+}]_i$, uncoupling of the contractile apparatus by activating myosin light chain phosphatase (MLCP) and hyperpolarization of the membrane (Archer et al. 1994; Koyama et al. 2001; Lu et al. 2010; Pauvert et al. 2004; Rybalkin and Bornfeldt 1999; Wang et al. 2009). Sildenafil downregulates the increased expression of TRPC1 and TRPC6 in PAH-PASMCs, thereby decreasing capacitative calcium entry (CCE) and inhibiting proliferation by ET-1 and 5-HT; inhibition of NFAT translocation and activation by sildenafil may be key for the decrease in TRPC expression (Lu et al. 2010; Pauvert et al. 2004; Wang et al. 2009). In parallel, PDE5 inhibition can also suppress RhoA activation and attenuate MMP2 production, mechanisms that contribute to decreased pulmonary vascular remodeling (Sun et al. 2009b). Inhibition of RhoA prevents ROCK-dependent inhibition of MLCP, thereby upregulating MKP-1 that can also decrease phosphorylation of ERK1/2 and suppress PSMC proliferation (Guilluy et al. 2005). In vascular smooth muscle cells, sildenafil activates RGS2 (the regulator of G-protein-coupled signaling 2) to suppress Gq-mediated contraction; if such an effect occurs in PSMCs, it could contribute to the blunting of ET-1 or 5-HT signaling by PDE5 inhibition (Lu et al. 2010; Pauvert et al. 2004; Takimoto et al. 2009; Wang et al. 2009). Many studies imply that some of the beneficial effects of PDE5-inhibitors are, at least in part, mediated by an increase in cAMP, which results from an inhibition of PDE3 (a cGMP-inhibited PDE) by the increased levels of cGMP, as will be discussed below (Koyama et al. 2001; Zaccolo and Movsesian 2007). However, we find that the acute stimulation of PAH-PASMCs with zaprinast does not increase the blunting of agonist-induced cAMP accumulation in these cells (Fig. 2b).

Role of PDE3 in Pulmonary Vasculature

PDE3 is a major PDE in the lung and both PDE3A and PDE3B are expressed in human and rat PSMCs (Murray et al. 2002, 2007; Palmer and Maurice 2000). The mRNA and protein expression and activity of PDE3 is increased in animal models of PAH and PAH-PASMCs (PDE3A and PDE3B, Fig. 1b, c) and after exposure of PSMCs to chronic hypoxia (which, in particular, enhances expression of PDE3A (Murray et al. 2002, 2007; Wagner et al. 1997). Elevated PDE3 activity, at least in part, would explain reduced $[\text{cAMP}]_i$ in both isolated lung from the chronic-hypoxic rat and agonist-induced cAMP accumulation in PAH-PASMCs; PDE3

inhibition increases beraprost (a stable analogue of prostacyclin)-induced cAMP accumulation from 6 ± 1 to 47 ± 1 pmol/mg in PAH-PASMCs (Fig. 2b) (MacLean et al. 1996, 1997; Murray et al. 2007). Increased PDE3 activity contributes to the decrease response to cAMP-elevating agents, such as isoproterenol and forskolin, in vasodilating the PA from chronic hypoxic rats and decreasing proliferation of PAH-PASMCs (Murray et al. 2007; Pyne et al. 2007; Schermuly et al. 2005). The PDE3 inhibitor milrinone decreases proliferation in PAH-PASMCs to a similar degree as does PDE5 inhibition (Fig. 2a). PDE3 appears important in maintaining the SMC phenotype in PASMCs, such that PDE3 activity and PDE3A expression are reduced in synthetic/activated cells (Dunkerley et al. 2002). PDE3 inhibitors, such as milrinone or SCA40, relax precontracted PA rings from both control- and PAH-rats, providing evidence that PDE3 activity may help maintain the low tone of the pulmonary circulation (Jeffery and Wanstall 1998; Pyne et al. 2007; Wagner et al. 1997). Figure 2c, d show that SK9483 promotes relaxation of both the main and first branch PA precontracted with phenylephrine.

Importantly, the effects of the PDE3 inhibitors are not compromised in PAH. In animal models of PAH, amrinone, cilostamide, milrinone, and cilostazol (Pletal) have all been shown to decrease mPAP and PVR and even reverse remodeling of the PA (Clarke et al. 1994; Phillips et al. 2005; Sun et al. 2009a). Cilostazol (Pletal), which is FDA approved for intermittent claudication, partially reverses PAH in animal models of the disease and acts synergistically with bone marrow-derived endothelial progenitor cells to inhibit the inflammatory response that is seen in such models (Sun et al. 2009a; Thompson et al. 2002). Although PDE3 inhibitors effectively lower PA wedge pressure and mPAP, because they have positive inotropic and vasodilatory actions in the systemic circulation, they were deemed an unsuitable option for treating PAH. In addition, based on results of clinical trials for heart failure that showed PDE3 inhibitors increased mortality by around 30% (Packer et al. 1991), there has been a reluctance to consider their use in PAH.

However, PDE3 is being “revisited” as a possible target for PAH because PDE3 inhibition may have therapeutic potential either in combination with approved drugs such as prostacyclin and its analogues or even PDE5 inhibitors (allowing the use of lower doses with less cardiovascular side effects), by altering the route of administration or only using the PDE3 inhibitors in a subset of patients. Combination therapy for PAH with PDE3 inhibitors can produce potent and selective pulmonary vasodilation; the coapplication of subthreshold doses of PDE3 inhibitors amplify prostacyclin, isoproterenol, and forskolin-induced vasodilation of isolated PA from chronic-hypoxic rats, and attenuate PASMC proliferation (Phillips et al. 2005; Schermuly et al. 2001; Wagner et al. 1997). Distal pulmonary artery muscularization in the chronic-hypoxic rat is inhibited by the combination of iloprost and cilostamide (more than either agent alone), providing a rationale for their coadministration in reversing pulmonary vascular remodeling.

A similar interaction has been observed with PDE3 and PDE5 inhibitors, such that “a low dose” of each type of inhibitor yielded ~4 and 8% reduction of elevated PVR, respectively; however, together they reduced PVR by ~40%, implying a synergistic effect of their coadministration in reducing elevated PVR

(Clarke et al. 1994). A combination of milrinone and sildenafil achieved a better hemodynamic profile in a porcine model of acute PAH, with greater pulmonary vasodilatation and increased contractility but without additional systemic vasodilatation (Lobato et al. 2006). Furthermore, inhaled NO enhanced the dose-dependent decrease in PVR by milrinone by a further 20% (Deb et al. 2000). Inhalation of nebulized milrinone can improve the hemodynamic profile in animal models of PAH and patients with pulmonary hypertension secondary to congestive heart failure, suggesting this may be a novel, effective pulmonary-selective strategy; however, longer-term studies are required (Botha et al. 2009; Hentschel et al. 2007; Lamarche et al. 2007).

Inhibiting PDE3 increases cAMP, which decreases vasoconstriction and proliferation through a number of mechanisms, although which are necessary and sufficient for the beneficial effect of cAMP in PASMCs are not fully defined (Koyama et al. 2001; Rybalkin and Bornfeldt 1999). cAMP, via PKA activation, phosphorylates MLCK, leading to vasodilation of the PA and inhibits Raf-1 activation, thereby inhibiting ERK1/2 activation and PI3K, both of which attenuate PASMC proliferation. In smooth muscle cells, cAMP blocks cell cycle progression from G1 to S phase, inhibits cyclin-dependent kinase 4, and upregulates the cyclin-dependent kinase 2 inhibitor, p27kip1 (Koyama et al. 1996). In PAH-PASMCs, milrinone inhibited CCE ~50% (Murray et al. 2007). Inhibitors of PDE3 open BK_{Ca} channels in PAH-PASMCs, leading to membrane hyperpolarization, and also block the vasoconstrictive response to PKC activation in isolated PAH-PA (Barman et al. 2003; Zhu et al. 2008). The latter effects were shown to be independent of PKA, thereby highlighting a possible role for the other cAMP downstream mediators, such as Epac, in cAMP-promoted vasodilation and antiproliferation. Epac, a guanine nucleotide exchange factor for the low-molecular-weight G protein Rap-1, has been shown to mediate a number of effects of cAMP; our unpublished data show that PDE3 inhibition can activate Epac and that Epac activation decreases proliferation of both control- and PAH-PASMCs. Of note, PDE3B can integrate into a complex with Epac (Raymond et al. 2007), suggesting that if such an interaction occurs in PASMCs, increased cAMP that results from PDE3 inhibition might preferentially enhance Epac-dependent functions.

The notion that specific PDE isoforms can regulate distinct “pools” of cAMP could be key for the pharmacological actions of specific PDE inhibitors (Raymond et al. 2007). In addition, PKA-independent effects of PDE3 inhibitors conceivably may be due to cross-activation by cAMP of PKG (which is known to occur in numerous systems) or from cAMP-promoted increases in [cGMP]_i as a consequence of inhibition of its hydrolysis by interaction with dual-specificity PDEs (Koyama et al. 2001; Maurice 2005; Zaccolo and Movsesian 2007): such an idea is worth further investigation in PAH-PASMCs. It can be hypothesized that a potential way to limit the detrimental effects of PDE3 inhibitors may be targeting drugs to particular PDE3 isoforms (e.g., PDE3A or PDE3B), which based on their different subcellular distribution and possibly different downstream mediators, such inhibitors might have differential effects in the cell; currently, no such drugs are available.

3.1.2 Role of PDE1 in the Pulmonary Vasculature

Real-time PCR has demonstrated that a number of other PDEs are expressed in both whole lung and human PASMCs (Fig. 1a), although their roles in PAH have not been fully investigated. By defining the expression of PDEs in human PASMCs from normal subjects and patients with PAH, we obtained evidence for a biochemically and physiologically important role of PDE1 in PAH. PDE1 is encoded by three separate genes with several splice variants: PDE1A, PDE1B (which is absent in human PASMCs), and PDE1C, which hydrolyzes cAMP and cGMP with different affinities and mediates, at least in part, the decrease in cAMP accumulation in response to increases in $[Ca^{2+}]_i$ (Goraya and Cooper 2005; Miller et al. 2009). PDE1C has equal affinity for cAMP and cGMP and also hydrolyzes both at roughly equal rate, whereas PDE1A has ~20-fold higher affinity for cGMP and PDE1B has ~5-fold greater affinity for cGMP (Bender and Beavo 2006). PDE1 may thus provide “cross-talk” between the increased $[Ca^{2+}]_i$ and decreased levels of cyclic nucleotides that occur with PAH and unlike PDE5 or PDE3 inhibitors, inhibition of PDE1 would increase both $[cAMP]_i$ and $[cGMP]_i$, depending on the isoform that is most abundant in that tissue.

We found an increase in the mRNA and protein expression of PDE1A and PDE1C and increased total PDE1 activity in PASMCs isolated from patients with both primary and secondary forms of PAH compared to PASMCs from controls, and this increase contributed to decreased cAMP and cGMP levels and increased proliferation of PAH-PASMCs (Murray et al. 2007). Moreover, PDE1 inhibition reduced proliferation in PAH-PASMCs more than did inhibition of PDE2 or PDE4, decreased CCE by >50% and helped restore forskolin- and beraprost-induced cAMP accumulation (Fig. 2a, b). Expression of PDE1A, 1B, and 1C are detected in PA from mice, rat, and lamb and increased expression is observed after development of PAH (Evgenov et al. 2006; Schermuly et al. 2007). Figure 3 demonstrates that PDE1 expression and activity are increased in the monocrotaline-treated rat lung, providing a further rationale for targeting PDE1, in particular PDE1C, in PAH.

The data obtained from animal models that implicate a role for PDE1 in PAH must be interpreted with caution. In human SMCs, unlike SMCs from other species, PDE1C is more dependent on calmodulin-stimulated cAMP hydrolysis and an increase in PDE1C is associated with SMC proliferation (Rybalkin and Bornfeldt 1999; Rybalkin et al. 1997, 2002). Due to the marked differences in expression, activities, and function of PDE1 isoforms in SMCs from different species, animal models of PAH may not be appropriate to study PDE1 and thus, not show the full therapeutic potential of PDE1 inhibitors. Even so, in perfused rat lung preparations, administration of the PDE1 inhibitor vinpocetine attenuates acute hypoxic vasoconstriction and PDE1 inhibitors augment the therapeutic efficacy of inhaled NO and iloprost in animal models of PAH: PDE1 inhibitors applied at subthreshold doses enhanced iloprost-induced decrease in mPAP to a similar degree as a PDE3 inhibitor and did not decrease systemic pressure (Evgenov et al. 2006; Phillips et al. 2005; Schermuly et al. 2005; Wagner et al. 1997). The PDE inhibitor

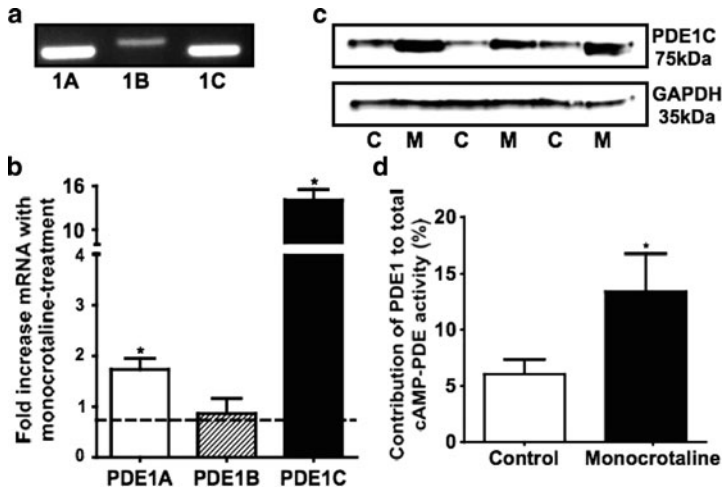


Fig. 3 PDE1 expression in rat lung and upregulation with PAH. (a) mRNA for PDE1A, 1B, and 1C is detected in rat lung. (b) PDE1A and PDE1C mRNA, (c) PDE1C protein, and (d) % contribution of PDE1 to total PDE1 activity is increased in monocrotaline-treated rat lung (M) relative to control (C). PDE activity was determined using 1 μ M cAMP as substrate through a two-step radioassay procedure (Keravis et al. 2005; Thompson et al. 1974). To identify the contribution of activity of PDE1, we performed assays in the presence of specific PDE1 inhibitors [30 μ M vinpocetine, 30 μ M 8-methoxy-methyl-3-isobutyl-1-methylxanthine (8-MM-IBMX)] and with or without Ca^{2+} /Calmodulin in the presence of EGTA. $n = 3$, $*P < 0.05$ vs. control

8MM-IBMX decreased PVR (2.14 ± 0.26 to 1.32 ± 0.33 mmHg mL⁻¹ min⁻¹ per 100 g of body weight) in the monocrotaline-treated rat and increased the portion of nonmuscularized PA in the chronic-hypoxic mouse (Schermuly et al. 2007). However, since both vinpocetine and 8MM-IBMX can inhibit other PDEs (in particular PDE7 and PDE5, respectively), this could alter the interpretation of the data.

Due to the lack of specific pharmacological inhibitors for PDE1, no clinical trials for PAH have been undertaken. However, vinpocetine (up to 40 mg per day) has been used to decrease smooth muscle tone in the lower urinary tract for urge incontinence and is approved and used in Eastern Europe to enhance cerebral blood-flow and improve memory (Kemeny et al. 2005; Truss et al. 2001). Although one must be cautious in drawing conclusions from such results, the studies suggest that PDE1 inhibitors are generally well tolerated. Furthermore, PDE5 inhibitors such as zaprinast and sildenafil interact with PDE1 (sildenafil has a IC₅₀ of 350 nM) and 100 mg sildenafil can lead to a plasma level of 1.2 μ M, suggesting that potentially some of the beneficial effects of “PDE5 inhibitors” in PAH may, in fact, result from inhibition of PDE1 (Paul et al. 2005). The data above highlight the need to test selective PDE1 inhibitors since their use is crucial to define the function of PDE1 and its potential as a therapeutic target for PAH.

Previous studies regarding specific PDE1 isoforms provide further evidence for a role of PDE1 in PAH, a disease characterized by reduced sensitivity to vasodilators and excess number and activity of smooth muscle cells. An increase in PDE1A

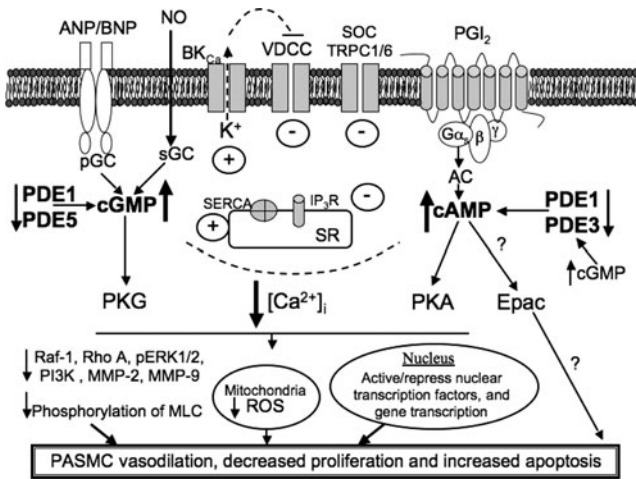


Fig. 4 Schematic of some mechanisms that contribute to the vasodilatory, antiproliferative, and apoptotic actions of cAMP and cGMP (by PDE inhibition) in PASMCs. The effects of cyclic nucleotides are mediated by PKG and PKA activation, potassium channel opening, and a decrease in intracellular $[Ca^{2+}]_i$ that leads to phosphorylation of a number of downstream proteins, decrease in ROS and altered activity of transcription factors. Further details are given in the text

expression has been shown to contribute to a reduced response (“tolerance”) to drugs that increase NO; the PDE1 inhibitor vinpocetine partially restores sensitivity of the vasculature to nitroglycerin (Kim et al. 2001). Moreover, in vascular SMCs, the nuclear localization of PDE1A can, by decreasing cGMP, protect the cells from apoptosis (Nagel et al. 2006). PDE1C is present in proliferating aortic SMCs but absent in intact human thoracic aorta and not expressed in contractile/quiescent cells, findings that suggest PDE1C contributes to the proliferative phenotype of vascular SMCs (Rybalkin and Bornfeldt 1999; Rybalkin et al. 1997, 2002); PDE1C has also been shown to associate with chromatin, which may contribute to SMC replication (Dolci et al. 2006). These data suggest that PDE1 isoforms regulate smooth muscle function and may be useful targets for the treatment of diseases with excess smooth muscle cell proliferation. Such ideas are consistent with our data showing that treatment with PDE1C-siRNA inhibits proliferation of PASMCs, in particular cells from PAH patients (Murray et al. 2007). Figure 4 shows a schematic of the signalling pathways and mediators that may contribute to the vasodilatory, antiproliferative and apoptotic actions of PDE inhibitors in PASMCs and hence their beneficial effects in PAH.

3.2 Role of PDEs in the Right Ventricular Hypertrophy

Much of the research in PAH focuses on developing effective treatments to vasodilate and reverse the remodeling of the PA and thereby reduce mPAP.

However, since the main cause of morbidity and mortality in PAH is right ventricular (RV) failure, and there is limited access to lung transplantation, future therapeutic approaches must seek to preserve RV function but knowledge is incomplete regarding the mechanisms for RV failure. In some patients, failure of the RV progresses even when mPAP is attenuated (Badesch et al. 2009; Bogaard et al. 2009). RV hypertrophy is a protective mechanism by which the heart compensates for the increase in PAP, however sustained, high pulmonary pressure leads to maladaptive remodeling, dilatation, and eventually RV failure. PDE1A, 1C, 3A, 3B, and 5A are all expressed in the heart and PDE family-specific inhibitors enhance contractility and ameliorate LV hypertrophy, although given the difference in the structure and function of the ventricles this may not be true for RV hypertrophy (Miller et al. 2009; Takimoto et al. 2005; Yan et al. 2007; Zaccolo and Movsesian 2007). Both in experimental and in clinical *in vivo* studies (referenced above), sildenafil and milrinone reverse RV hypertrophy, suggesting that in addition to improving the hemodynamics of the pulmonary circulation, PDE inhibitors help preserve RV function, by enhancing contractility and increasing cardiac output.

Limited data exist as to whether the attenuation of RV hypertrophy by PDE inhibition is due to a direct cardiac effect or the result of a decrease in mPAP. Sildenafil significantly increases contractility in both the perfused heart and the isolated cardiomyocytes with RV hypertrophy; PDE5, but not PDE3, mRNA, protein and activity are markedly upregulated in hypertrophied RV myocardium (Nagendran et al. 2007). In contrast, sildenafil does not reverse or prevent RV hypertrophy when the pressure load is kept constant by surgical banding of the PA, suggesting that PDE5 inhibitors act indirectly to decrease RV hypertrophy via RV unloading (Andersen et al. 2008; Schafer et al. 2009).

In isolated heart and myocytes, PDE5 inhibitors do not enhance PKG signaling but instead increase [cAMP]; (Nagendran et al. 2007). It has been proposed that increased cGMP (via sildenafil) competitively inhibits PDE3, thereby elevating cAMP since PKA inhibitors block the positive inotropic effect of sildenafil and no increase in PKG phosphorylation is observed in the RV after treatment with PDE5 inhibitors (Mouchaers et al. 2009; Nagendran et al. 2007). Sildenafil also improves NOS coupling and decreases superoxide levels (which participate in the transition from hypertrophy to heart failure) in the heart, thus contributing to its beneficial effects in the RV (Hemnes et al. 2008). Treatment of monocrotaline-treated rats with bosentan and sildenafil reduces the formation of fibrosis by increasing the activity of mitochondrial enzymes (succinate dehydrogenase and cytochrome *c* oxidase) in the RV (Hemnes et al. 2008; Mouchaers et al. 2009). More research is needed to determine whether components that contribute to the protective effect of PDE inhibitors on LV failure, such as RGS2, are also important in RV failure and whether their short-term benefits can be translated into long-term clinical utility.

3.3 Role of PDEs in the Inflammation Associated with PAH

Another aspect of PAH that may be targeted by PDE inhibition is the inflammatory component of the disease. Inflammatory cells such as B- and T cells, dendritic cells (DCs) and macrophages are all present in the vascular lesions of patients with IPAH or secondary PAH and in animal models such as the monocrotaline-treated rat (Hassoun et al. 2009; Tuder et al. 1994). PAH is associated with increased circulating levels of inflammatory mediators such as IL-1 β and IL-6 and cytokines and chemokines such as CX3CL1, CCL2, and CCL5 (Balabanian et al. 2002; Humbert et al. 1995; Perros et al. 2007). Release of these mediators from pulmonary endothelial and PASMC recruit inflammatory cells is hypothesized to initiate the release of growth factors, increase $[Ca^{2+}]_i$ and transcription and thereby, to increase remodeling of the PA. Other data have shown that PDE3 and PDE4 regulate cAMP accumulation and proliferation in human CD4+ and CD8+ T-lymphocytes and affect DC function by blocking TNF release, providing evidence that PDE3- and PDE4-inhibitors modulate inflammation [as discussed in other chapters, (Gantner et al. 1999; Giembycz et al. 1996)].

We have shown that the highest expressed PDEs in peripheral blood mononuclear cells (PBMCs) are PDE4B, PDE7A, and PDE3B (Zhang et al. 2008). PDE4, PDE3, and PDE7 contribute 40, 30, and 15%, respectively, to total-PDE activity in PASMCs, as determined through a two-step radioassay conducted in the absence or presence of selective PDE inhibitors. In addition to increasing cAMP in PASMCs (Millen et al. 2006; Murray et al. 2007; Schermuly et al. 2005), PDE4 inhibitors are important in modulating the action of immune and inflammatory cells (Souness et al. 2000), suggesting that PDE4 inhibitors may help decrease PASMC contraction and proliferation and also inhibit inflammation that contributes to the development of PAH. PDE4A10, PDE4A11, PDE4B2, PDE4C, and PDE4D5 isoforms are expressed in human PASMCs (Millen et al. 2006). Of note, the PDE4 inhibitor piclamilast inhibits proliferation of pulmonary fibroblasts induced by the basic fibroblast growth factor (bFGF) and low-dose interleukin-1 (IL-1 β), suggesting that it may prevent inflammation-induced remodeling of the PA (Selige et al. 2010). In vivo the PDE4 inhibitor roflumilast decreased mPAP, RV hypertrophy, and PA muscularization, thereby attenuating the development of monocrotaline-induced PAH. Roflumilast reduced the increases in IL-6 and monocyte chemoattractant protein-1 (MCP-1) that was observed in lung tissue after 21 days of monocrotaline-treatment and reversed established monocrotaline-induced PAH, thereby suggesting that blunting of inflammation associated with PAH can help attenuate PA remodeling (Izikki et al. 2009).

Since inhibition of PDE4 alone, compared to PDE3 and PDE1, only modestly decreases PASMC proliferation and increases cAMP accumulation in PAH-PASMC (Fig. 2a, b), dual PDE3/PDE4 inhibitors may be a rational therapeutic

approach by targeting both the vascular and the immune aspects of the disease (Fig. 1) (Izikki et al. 2009). In the monocrotaline-treated rat, in which inflammation is key to the development of PAH, the PDE3/PDE4 inhibitor tolafentrine alone or together with iloprost reverses the increase in mPAP, RV hypertrophy, and pulmonary vascular remodeling in fully established PAH and inhibits the associated increase in activity of MMP-2 and MMP-9: although systemic arterial pressure decreases on acute exposure to tolafentrine, this is not sustained (Schermuly et al. 2004). In parallel, the dual PDE3/PDE4 inhibitor pumafentrine, which dose-dependently increases cAMP and decreases proliferation in isolated human PASMCs, significantly reversed the muscularization, media hypertrophy, and the decrease in the lumen of the small PAs, suggesting that this inhibitor is proapoptotic (Dony et al. 2008). These data imply that targeting PDE4/PDE3 would be beneficial for PAH. However, due to the dose-limiting side effects of PDE3 and PDE4 inhibitors, more research is required to determine whether such drugs will be clinically useful. Because PDE7 is also highly expressed in immune cells and upregulated in PAH-PASMCs, targeting of this PDE could be a novel approach in the treatment of PAH and merits further investigation.

4 Conclusion and the Future of PDEs in PAH

PAH is a complex, multifactorial disease with no single cause and thus, it is unlikely that one drug will prove to be therapeutically beneficial for all patients. Nevertheless, the evidence reviewed here strongly implicates a key role for PDEs in the increased mPAP and remodeling of the PA that is associated with PAH. The clinical success of PDE5 inhibitors (e.g., sildenafil) in different forms of PAH implies that PDE5 expression and activity contribute to the pathophysiology of PAH. In order to more clearly determine the role of other PDEs, in particular PDE1, selective inhibitors are needed; the development of inhibitors that can target specific PDE isoforms has the potential to provide therapeutic utility by providing efficacy but decreasing side effects.

Since mRNA for PDE1A, PDE1C, PDE3A, PDE3B, and PDE5A are all upregulated in PAH, their regulation in this disease likely occurs at the level of transcription (Fig. 1b) (Murray et al. 2007). However, relatively limited information is available regarding the transcriptional control of specific PDEs. We have hypothesized that the increase in PDE3A expression with PAH is attributable to PKA-dependent CREB activation, through an initial accumulation of cAMP to protect the pulmonary circulation from insult and to try and restore normal tone (Murray et al. 2002). Studies have suggested that the increase in PDE5A mRNA occurs via increased NF κ B activation; mapping the promoter of PDE5A identified a number of Sp1-, AP-1, NF κ B-, and CRE-binding sites (Lin et al. 2001a, b; Murray et al. 2002; Pyne et al. 2007). Further data show that PDE5A expression is upregulated via Ang-II-mediated ERK1/2-activation and increased superoxide derived from NOX; however, no transcription factors were directly implicated

(Kim et al. 2005; Muzaffar et al. 2008). Data supporting the role of one or more transcription factors in the regulation of PDEs in PAH would likely advance understanding of PDEs and perhaps identify mechanisms that contribute to the initiation and progression of PAH.

Real-time PCR and immunoblot analyses have demonstrated that a number of other PDEs and PDE-related proteins, including the two isoforms of the small inhibitory proteins PDE γ 1 and PDE γ 2, which were first shown to be associated with PDE6 regulation in photoreceptor cells, are expressed in PASMCs; however, they have not been shown to regulate the PDEs expressed in PASMCs and their function and relevance to PAH have not been fully investigated (Fig. 1a, Murray et al. 2003, 2007). These or other PDEs may be highly expressed in the RV or in inflammatory cells such as lymphocytes, macrophages, and DCs that infiltrate the PA and may be as important to PAH as those outlined above. Further analyses of the regulation, expression, and function of PDEs in the pulmonary circulation and the effectiveness of combination therapy with PDE inhibitors would increase the understanding of PAH and may aid in the development of novel therapeutic targets or regimens.

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References

- Ahn HS, Foster M, Cable M, Pitts BJ, Sybertz EJ (1991) Ca/CaM-stimulated and cGMP-specific phosphodiesterases in vascular and non-vascular tissues. *Adv Exp Med Biol* 308:191–197
- Andersen A, Nielsen JM, Peters CD, Schou UK, Sloth E, Nielsen-Kudsk JE (2008) Effects of phosphodiesterase-5 inhibition by sildenafil in the pressure overloaded right heart. *Eur J Heart Fail* 10:1158–1165
- Archer SL, Huang JM, Hampl V, Nelson DP, Shultz PJ, Weir EK (1994) Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci USA* 91:7583–7587
- Badesch DB, Champion HC, Sanchez MA, Hooper MM, Loyd JE, Manes A, McGoon M, Naeije R, Olschewski H, Oudiz RJ, Torbicki A (2009) Diagnosis and assessment of pulmonary arterial hypertension. *J Am Coll Cardiol* 54:S55–S66
- Balabanian K, Foussat A, Dorfmueller P, Durand-Gasselin I, Capel F, Bouchet-Delbos L, Portier A, Marfaing-Koka A, Krzysiek R, Rimaniol AC, Simonneau G, Emilie D, Humbert M (2002) CX(3)C chemokine fractalkine in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 165:1419–1425
- Baliga RS, Zhao L, Madhani M, Lopez-Torondel B, Visintin C, Selwood D, Wilkins MR, MacAllister RJ, Hobbs AJ (2008) Synergy between natriuretic peptides and phosphodiesterase 5 inhibitors ameliorates pulmonary arterial hypertension. *Am J Respir Crit Care Med* 178:861–869
- Barman SA, Zhu S, Han G, White RE (2003) cAMP activates BKCa channels in pulmonary arterial smooth muscle via cGMP-dependent protein kinase. *Am J Physiol Lung Cell Mol Physiol* 284:L1004–L1011

- Barnes PJ, Liu SF (1995) Regulation of pulmonary vascular tone. *Pharmacol Rev* 47:87–131
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Black SM, Sanchez LS, Mata-Greenwood E, Bekker JM, Steinhorn RH, Fineman JR (2001) sGC and PDE5 are elevated in lambs with increased pulmonary blood flow and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 281:L1051–L1057
- Bogaard HJ, Abe K, Vonk Noordegraaf A, Voelkel NF (2009) The right ventricle under pressure: cellular and molecular mechanisms of right-heart failure in pulmonary hypertension. *Chest* 135:794–804
- Botha P, Parry G, Dark JH, Macgowan GA (2009) Acute hemodynamic effects of intravenous sildenafil citrate in congestive heart failure: comparison of phosphodiesterase type-3 and -5 inhibition. *J Heart Lung Transplant* 28:676–682
- Chaouat A, Coulet F, Favre C, Simonneau G, Weitzenblum E, Soubrier F, Humbert M (2004) Endoglin germline mutation in a patient with hereditary haemorrhagic telangiectasia and dexfenfluramine associated pulmonary arterial hypertension. *Thorax* 59:446–448
- Clarke WR, Uezono S, Chambers A, Doepfner P (1994) The type III phosphodiesterase inhibitor milrinone and type V PDE inhibitor dipyridamole individually and synergistically reduce elevated pulmonary vascular resistance. *Pulm Pharmacol* 7:81–89
- Cohen AH, Hanson K, Morris K, Fouty B, McMurty IF, Clarke W, Rodman DM (1996) Inhibition of cyclic 3'-5'-guanosine monophosphate-specific phosphodiesterase selectively vasodilates the pulmonary circulation in chronically hypoxic rats. *J Clin Invest* 97:172–179
- Corbin JD, Beasley A, Blount MA, Francis SH (2005) High lung PDE5: a strong basis for treating pulmonary hypertension with PDE5 inhibitors. *Biochem Biophys Res Commun* 334:930–938
- D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Kernis JT et al (1991) Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann Intern Med* 115:343–349
- Deb B, Bradford K, Pearl RG (2000) Additive effects of inhaled nitric oxide and intravenous milrinone in experimental pulmonary hypertension. *Crit Care Med* 28:795–799
- Dempse Y, Morecroft I, Welsh DJ, MacRitchie NA, Herold N, Loughlin L, Nilsen M, Peacock AJ, Harmar A, Bader M, MacLean MR (2008) Converging evidence in support of the serotonin hypothesis of dexfenfluramine-induced pulmonary hypertension with novel transgenic mice. *Circulation* 117:2928–2937
- Dent G, Magnussen H, Rabe KF (1994) Cyclic nucleotide phosphodiesterases in the human lung. *Lung* 172:129–146
- Dolci S, Belmonte A, Santone R, Giorgi M, Pellegrini M, Carosa E, Piccione E, Lenzi A, Jannini EA (2006) Subcellular localization and regulation of type-1C and type-5 phosphodiesterases. *Biochem Biophys Res Commun* 341:837–846
- Dony E, Lai YJ, Dumitrascu R, Pullamsetti SS, Savai R, Ghofrani HA, Weissmann N, Schudt C, Flockerzi D, Seeger W, Grimminger F, Schermuly RT (2008) Partial reversal of experimental pulmonary hypertension by phosphodiesterase-3/4 inhibition. *Eur Respir J* 31:599–610
- Dunkerley HA, Tilley DG, Palmer D, Liu H, Jimmo SL, Maurice DH (2002) Reduced phosphodiesterase 3 activity and phosphodiesterase 3A level in synthetic vascular smooth muscle cells: implications for use of phosphodiesterase 3 inhibitors in cardiovascular tissues. *Mol Pharmacol* 61:1033–1040
- Eddahibi S, Raffestin B, Monnier L, de Gouville AC, Adnot S (1998) Effect of DMPPQ, a phosphodiesterase type 5 inhibitor, on hypoxic pulmonary hypertension in rats. *Br J Pharmacol* 125:681–688
- Evgenov OV, Busch CJ, Evgenov NV, Liu R, Petersen B, Falkowski GE, Petho B, Vas A, Bloch KD, Zapol WM, Ichinose F (2006) Inhibition of phosphodiesterase 1 augments the pulmonary vasodilator response to inhaled nitric oxide in awake lambs with acute pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 290:L723–L729

- Fink TL, Francis SH, Beasley A, Grimes KA, Corbin JD (1999) Expression of an active, monomeric catalytic domain of the cGMP-binding cGMP-specific phosphodiesterase (PDE5). *J Biol Chem* 274:34613–34620
- Francis SH, Chu DM, Thomas MK, Beasley A, Grimes K, Busch JL, Turko IV, Haik TL, Corbin JD (1998) Ligand-induced conformational changes in cyclic nucleotide phosphodiesterases and cyclic nucleotide-dependent protein kinases. *Methods* 14:81–92
- Galie N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzyna M, Simonneau G (2005) Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med* 353:2148–2157
- Galie N, Brundage BH, Ghofrani HA, Oudiz RJ, Simonneau G, Safdar Z, Shapiro S, White RJ, Chan M, Beardsworth A, Frumkin L, Barst RJ (2009a) Tadalafil therapy for pulmonary arterial hypertension. *Circulation* 119:2894–2903
- Galie N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, Barbera JA, Beghetti M, Corris P, Gaine S, Gibbs JS, Gomez-Sanchez MA, Jondeau G, Klepetko W, Opitz C, Peacock A, Rubin L, Zellweger M, Simonneau G (2009b) Guidelines for the diagnosis and treatment of pulmonary hypertension. *Eur Respir J* 34:1219–1263
- Galie N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, Barbera JA, Beghetti M, Corris P, Gaine S, Gibbs JS, Gomez-Sanchez MA, Jondeau G, Klepetko W, Opitz C, Peacock A, Rubin L, Zellweger M, Simonneau G, Vahanian A, Auricchio A, Bax J, Ceconi C, Dean V, Filippatos G, Funck-Brentano C, Hobbs R, Kearney P, McDonagh T, McGregor K, Popescu BA, Reiner Z, Sechtem U, Sirnes PA, Tendera M, Vardas P, Widimsky P, Al Attar N, Andreotti F, Aschermann M, Asteggiano R, Benza R, Berger R, Bonnet D, Delcroix M, Howard L, Kitsiou AN, Lang I, Maggioni A, Nielsen-Kudsk JE, Park M, Perrone-Filardi P, Price S, Domenech MT, Vonk-Noordegraaf A, Zamorano JL (2009c) Guidelines for the diagnosis and treatment of pulmonary hypertension: The Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). *Eur Heart J* 30:2493–2537
- Gantner F, Schudt C, Wendel A, Hatzelmann A (1999) Characterization of the phosphodiesterase (PDE) pattern of in vitro-generated human dendritic cells (DC) and the influence of PDE inhibitors on DC function. *Pulm Pharmacol Ther* 12:377–386
- Ghofrani HA, Wiedemann R, Rose F, Olschewski H, Schermuly RT, Weissmann N, Seeger W, Grimminger F (2002) Combination therapy with oral sildenafil and inhaled iloprost for severe pulmonary hypertension. *Ann Intern Med* 136:515–522
- Ghofrani HA, Rose F, Schermuly RT, Olschewski H, Wiedemann R, Kreckel A, Weissmann N, Ghofrani S, Enke B, Seeger W, Grimminger F (2003) Oral sildenafil as long-term adjunct therapy to inhaled iloprost in severe pulmonary arterial hypertension. *J Am Coll Cardiol* 42:158–164
- Ghofrani HA, Voswinckel R, Reichenberger F, Olschewski H, Haredza P, Karadas B, Schermuly RT, Weissmann N, Seeger W, Grimminger F (2004) Differences in hemodynamic and oxygenation responses to three different phosphodiesterase-5 inhibitors in patients with pulmonary arterial hypertension: a randomized prospective study. *J Am Coll Cardiol* 44:1488–1496
- Giembycz MA, Corrigan CJ, Seybold J, Newton R, Barnes PJ (1996) Identification of cyclic AMP phosphodiesterases 3, 4 and 7 in human CD4+ and CD8+ T-lymphocytes: role in regulating proliferation and the biosynthesis of interleukin-2. *Br J Pharmacol* 118:1945–1958
- Horaya TA, Cooper DM (2005) Ca²⁺-calmodulin-dependent phosphodiesterase (PDE1): current perspectives. *Cell Signal* 17:789–797
- Grandoch M, Roscioni SS, Schmidt M (2010) The role of Epac proteins, novel cAMP mediators, in the regulation of immune, lung and neuronal function. *Br J Pharmacol* 159:265–284
- Guilluy C, Sauzeau V, Rolli-Derkinderen M, Guerin P, Sagan C, Pacaud P, Loirand G (2005) Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol* 146:1010–1018

- Hanasato N, Oka M, Muramatsu M, Nishino M, Adachi H, Fukuchi Y (1999) E-4010, a selective phosphodiesterase 5 inhibitor, attenuates hypoxic pulmonary hypertension in rats. *Am J Physiol* 277:L225–L232
- Hanson KA, Ziegler JW, Rybalkin SD, Miller JW, Abman SH, Clarke WR (1998) Chronic pulmonary hypertension increases fetal lung cGMP phosphodiesterase activity. *Am J Physiol* 275:L931–L941
- Hassoun PM, Mouthon L, Barbera JA, Eddahibi S, Flores SC, Grimminger F, Jones PL, Maitland ML, Michelakis ED, Morrell NW, Newman JH, Rabinovitch M, Schermuly R, Stenmark KR, Voelkel NF, Yuan JX, Humbert M (2009) Inflammation, growth factors, and pulmonary vascular remodeling. *J Am Coll Cardiol* 54:S10–S19
- Hemnes AR, Zaiman A, Champion HC (2008) PDE5A inhibition attenuates bleomycin-induced pulmonary fibrosis and pulmonary hypertension through inhibition of ROS generation and RhoA/Rho kinase activation. *Am J Physiol Lung Cell Mol Physiol* 294:L24–L33
- Hentschel T, Yin N, Riad A, Habbazettl H, Weimann J, Koster A, Tschope C, Kuppe H, Kuebler WM (2007) Inhalation of the phosphodiesterase-3 inhibitor milrinone attenuates pulmonary hypertension in a rat model of congestive heart failure. *Anesthesiology* 106: 124–131
- Hoepfer MM, Faulenbach C, Golpon H, Winkler J, Welte T, Niedermeyer J (2004) Combination therapy with bosentan and sildenafil in idiopathic pulmonary arterial hypertension. *Eur Respir J* 24:1007–1010
- Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, Galanaud P, Simonneau G, Emilie D (1995) Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. *Am J Respir Crit Care Med* 151: 1628–1631
- Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M (2004) Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* 43:13S–24S
- Humbert M, Sitbon O, Chaouat A, Bertocchi M, Habib G, Gressin V, Yaici A, Weitzenblum E, Cordier JF, Chabot F, Dromer C, Pison C, Reynaud-Gaubert M, Haloun A, Laurent M, Hachulla E, Simonneau G (2006) Pulmonary arterial hypertension in France: results from a national registry. *Am J Respir Crit Care Med* 173:1023–1030
- Ichinose F, Adrie C, Hurford WE, Bloch KD, Zapol WM (1998) Selective pulmonary vasodilation induced by aerosolized zaprinast. *Anesthesiology* 88:410–416
- Izikki M, Raffestin B, Klar J, Hatzelmann A, Marx D, Tenor H, Zadigue P, Adnot S, Eddahibi S (2009) Effects of roflumilast, a phosphodiesterase-4 inhibitor, on hypoxia- and monocrotaline-induced pulmonary hypertension in rats. *J Pharmacol Exp Ther* 330:54–62
- Jeffery TK, Wanstall JC (1998) Phosphodiesterase III and V inhibitors on pulmonary artery from pulmonary hypertensive rats: differences between early and established pulmonary hypertension. *J Cardiovasc Pharmacol* 32:213–219
- Jing ZC, Jiang X, Wu BX, Xu XQ, Wu Y, Ma CR, Wang Y, Yang YJ, Pu JL, Gao W (2009) Vardenafil treatment for patients with pulmonary arterial hypertension: a multicentre, open-label study. *Heart* 95:1531–1536
- Kemeny V, Molnar S, Andrejkovics M, Makai A, Csiba L (2005) Acute and chronic effects of vinpocetine on cerebral hemodynamics and neuropsychological performance in multi-infarct patients. *J Clin Pharmacol* 45:1048–1054
- Keravis T, Thaseldar-Roumie R, Lugnier C (2005) Assessment of phosphodiesterase isozyme contribution in cell and tissue extracts. *Methods Mol Biol* 307:63–74
- Kim D, Rybalkin SD, Pi X, Wang Y, Zhang C, Munzel T, Beavo JA, Berk BC, Yan C (2001) Upregulation of phosphodiesterase 1A1 expression is associated with the development of nitrate tolerance. *Circulation* 104:2338–2343
- Kim D, Aizawa T, Wei H, Pi X, Rybalkin SD, Berk BC, Yan C (2005) Angiotensin II increases phosphodiesterase 5A expression in vascular smooth muscle cells: a mechanism by which angiotensin II antagonizes cGMP signaling. *J Mol Cell Cardiol* 38:175–184

- Klinger JR, Thaker S, Houtchens J, Preston IR, Hill NS, Farber HW (2006) Pulmonary hemodynamic responses to brain natriuretic peptide and sildenafil in patients with pulmonary arterial hypertension. *Chest* 129:417–425
- Kodama K, Adachi H (1999) Improvement of mortality by long-term E4010 treatment in monocrotaline-induced pulmonary hypertensive rats. *J Pharmacol Exp Ther* 290:748–752
- Koyama H, Raines EW, Bornfeldt KE, Roberts JM, Ross R (1996) Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. *Cell* 87:1069–1078
- Koyama H, Bornfeldt KE, Fukumoto S, Nishizawa Y (2001) Molecular pathways of cyclic nucleotide-induced inhibition of arterial smooth muscle cell proliferation. *J Cell Physiol* 186:1–10
- Lamarche Y, Perrault LP, Maltais S, Tetreault K, Lambert J, Denault AY (2007) Preliminary experience with inhaled milrinone in cardiac surgery. *Eur J Cardiothorac Surg* 31:1081–1087
- Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA 3rd, Loyd JE, Nichols WC, Trembath RC (2000) Heterozygous germline mutations in *BMPR2*, encoding a TGF- β receptor, cause familial primary pulmonary hypertension. *Nat Genet* 26:81–84
- Li M, Sun X, Li Z, Liu Y (2009) Inhibition of cGMP phosphodiesterase 5 suppresses serotonin signalling in pulmonary artery smooth muscles cells. *Pharmacol Res* 59:312–318
- Lin CS, Chow S, Lau A, Tu R, Lue TF (2001a) Identification and regulation of human PDE5A gene promoter. *Biochem Biophys Res Commun* 280:684–692
- Lin CS, Chow S, Lau A, Tu R, Lue TF (2001b) Regulation of human PDE5A2 intronic promoter by cAMP and cGMP: identification of a critical Sp1-binding site. *Biochem Biophys Res Commun* 280:693–699
- Lobato EB, Beaver T, Muehlschlegel J, Kirby DS, Klodell C, Sidi A (2006) Treatment with phosphodiesterase inhibitors type III and V: milrinone and sildenafil is an effective combination during thromboxane-induced acute pulmonary hypertension. *Br J Anaesth* 96:317–322
- Long L, Crosby A, Yang X, Southwood M, Upton PD, Kim DK, Morrell NW (2009) Altered bone morphogenetic protein and transforming growth factor- β signaling in rat models of pulmonary hypertension: potential for activin receptor-like kinase-5 inhibition in prevention and progression of disease. *Circulation* 119:566–576
- Lu W, Ran P, Zhang D, Peng G, Li B, Zhong N, Wang J (2010) Sildenafil inhibits chronically hypoxic upregulation of canonical transient receptor potential expression in rat pulmonary arterial smooth muscle. *Am J Physiol Cell Physiol* 298:C114–C123
- Machado RD, Pauciulo MW, Thomson JR, Lane KB, Morgan NV, Wheeler L, Phillips JA 3rd, Newman J, Williams D, Galie N, Manes A, McNeil K, Yacoub M, Mikhail G, Rogers P, Corris P, Humbert M, Donnai D, Martensson G, Tranebjaerg L, Loyd JE, Trembath RC, Nichols WC (2001) *BMPR2* haploinsufficiency as the inherited molecular mechanism for primary pulmonary hypertension. *Am J Hum Genet* 68:92–102
- Machado RD, Eickelberg O, Elliott CG, Geraci MW, Hanaoka M, Loyd JE, Newman JH, Phillips JA 3rd, Soubrier F, Trembath RC, Chung WK (2009) Genetics and genomics of pulmonary arterial hypertension. *J Am Coll Cardiol* 54:S32–S42
- MacLean MR, Sweeney G, Baird M, McCulloch KM, Houslay M, Morecroft I (1996) 5-Hydroxytryptamine receptors mediating vasoconstriction in pulmonary arteries from control and pulmonary hypertensive rats. *Br J Pharmacol* 119:917–930
- Maclean MR, Johnston ED, McCulloch KM, Pooley L, Houslay MD, Sweeney G (1997) Phosphodiesterase isoforms in the pulmonary arterial circulation of the rat: changes in pulmonary hypertension. *J Pharmacol Exp Ther* 283:619–624
- Maurice DH (2005) Cyclic nucleotide phosphodiesterase-mediated integration of cGMP and cAMP signaling in cells of the cardiovascular system. *Front Biosci* 10:1221–1228
- McLaughlin VV, Archer SL, Badesch DB, Barst RJ, Farber HW, Lindner JR, Mathier MA, McGoon MD, Park MH, Rosenson RS, Rubin LJ, Tapson VF, Varga J (2009) ACCF/AHA 2009 expert consensus document on pulmonary hypertension a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians;

- American Thoracic Society, Inc.; and the Pulmonary Hypertension Association. *J Am Coll Cardiol* 53:1573–1619
- Michelakis E, Tymchak W, Lien D, Webster L, Hashimoto K, Archer S (2002) Oral sildenafil is an effective and specific pulmonary vasodilator in patients with pulmonary arterial hypertension: comparison with inhaled nitric oxide. *Circulation* 105:2398–2403
- Millen J, MacLean MR, Houslay MD (2006) Hypoxia-induced remodelling of PDE4 isoform expression and cAMP handling in human pulmonary artery smooth muscle cells. *Eur J Cell Biol* 85:679–691
- Miller CL, Oikawa M, Cai Y, Wojtovich AP, Nagel DJ, Xu X, Xu H, Florio V, Rybalkin SD, Beavo JA, Chen YF, Li JD, Blaxall BC, Abe J, Yan C (2009) Role of Ca²⁺/calmodulin-stimulated cyclic nucleotide phosphodiesterase 1 in mediating cardiomyocyte hypertrophy. *Circ Res* 105:956–964
- Morrell NW, Adnot S, Archer SL, Dupuis J, Jones PL, MacLean MR, McMurtry IF, Stenmark KR, Thistlethwaite PA, Weissmann N, Yuan JX, Weir EK (2009) Cellular and molecular basis of pulmonary arterial hypertension. *J Am Coll Cardiol* 54:S20–S31
- Mouchaers KT, Schaliij I, Versteilen AM, Hadi AM, van Nieuw Amerongen GP, van Hinsbergh VW, Postmus PE, van der Laarse WJ, Vonk-Noordegraaf A (2009) Endothelin receptor blockade combined with phosphodiesterase-5 inhibition increases right ventricular mitochondrial capacity in pulmonary arterial hypertension. *Am J Physiol Heart Circ Physiol* 297:H200–H207
- Mullershausen F, Friebe A, Feil R, Thompson WJ, Hofmann F, Koesling D (2003) Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling. *J Cell Biol* 160:719–727
- Murray F, MacLean MR, Pyne NJ (2002) Increased expression of the cGMP-inhibited cAMP-specific (PDE3) and cGMP binding cGMP-specific (PDE5) phosphodiesterases in models of pulmonary hypertension. *Br J Pharmacol* 137:1187–1194
- Murray F, MacLean MR, Pyne NJ (2003) An assessment of the role of the inhibitory gamma subunit of the retinal cyclic GMP phosphodiesterase and its effect on the p42/p44 mitogen-activated protein kinase pathway in animal and cellular models of pulmonary hypertension. *Br J Pharmacol* 138:1313–1319
- Murray F, Patel HH, Suda RY, Zhang S, Thistlethwaite PA, Yuan JX, Insel PA (2007) Expression and activity of cAMP phosphodiesterase isoforms in pulmonary artery smooth muscle cells from patients with pulmonary hypertension: role for PDE1. *Am J Physiol Lung Cell Mol Physiol* 292:L294–L303
- Muzaffar S, Shukla N, Bond M, Sala-Newby GB, Newby AC, Angelini GD, Jeremy JY (2008) Superoxide from NADPH oxidase upregulates type 5 phosphodiesterase in human vascular smooth muscle cells: inhibition with iloprost and NONOate. *Br J Pharmacol* 155:847–856
- Nagel DJ, Aizawa T, Jeon KI, Liu W, Mohan A, Wei H, Miano JM, Florio VA, Gao P, Korshunov VA, Berk BC, Yan C (2006) Role of nuclear Ca²⁺/calmodulin-stimulated phosphodiesterase 1A in vascular smooth muscle cell growth and survival. *Circ Res* 98:777–784
- Nagendran J, Archer SL, Soliman D, Gurtu V, Moudgil R, Haromy A, St Aubin C, Webster L, Rebeyka IM, Ross DB, Light PE, Dyck JR, Michelakis ED (2007) Phosphodiesterase type 5 is highly expressed in the hypertrophied human right ventricle, and acute inhibition of phosphodiesterase type 5 improves contractility. *Circulation* 116:238–248
- Olschewski H, Simonneau G, Galie N, Higenbottam T, Naeije R, Rubin LJ, Nikkho S, Speich R, Hoepfer MM, Behr J, Winkler J, Sitbon O, Popov W, Ghofrani HA, Manes A, Kiely DG, Ewert R, Meyer A, Corris PA, Delcroix M, Gomez-Sanchez M, Siedentop H, Seeger W (2002) Inhaled iloprost for severe pulmonary hypertension. *N Engl J Med* 347:322–329
- Packer M, Carver JR, Rodeheffer RJ, Ivanhoe RJ, DiBianco R, Zeldis SM, Hendrix GH, Bommer WJ, Elkayam U, Kukin ML et al (1991) Effect of oral milrinone on mortality in severe chronic heart failure. The PROMISE Study Research Group. *N Engl J Med* 325:1468–1475
- Palmer D, Maurice DH (2000) Dual expression and differential regulation of phosphodiesterase 3A and phosphodiesterase 3B in human vascular smooth muscle: implications for phosphodiesterase 3 inhibition in human cardiovascular tissues. *Mol Pharmacol* 58:247–252

- Paul GA, Gibbs JS, Boobis AR, Abbas A, Wilkins MR (2005) Bosentan decreases the plasma concentration of sildenafil when coprescribed in pulmonary hypertension. *Br J Clin Pharmacol* 60:107–112
- Pauvert O, Bonnet S, Rousseau E, Marthan R, Savineau JP (2004) Sildenafil alters calcium signaling and vascular tone in pulmonary arteries from chronically hypoxic rats. *Am J Physiol Lung Cell Mol Physiol* 287:L577–L583
- Peacock AJ, Murphy NF, McMurray JJ, Caballero L, Stewart S (2007) An epidemiological study of pulmonary arterial hypertension. *Eur Respir J* 30:104–109
- Perros F, Dorfmueller P, Souza R, Durand-Gasselini I, Godot V, Capel F, Adnot S, Eddahibi S, Mazmanian M, Fadel E, Herve P, Simonneau G, Emilie D, Humbert M (2007) Fractalkine-induced smooth muscle cell proliferation in pulmonary hypertension. *Eur Respir J* 29:937–943
- Phillips PG, Long L, Wilkins MR, Morrell NW (2005) cAMP phosphodiesterase inhibitors potentiate effects of prostacyclin analogs in hypoxic pulmonary vascular remodeling. *Am J Physiol Lung Cell Mol Physiol* 288:L103–L115
- Pyne NJ, Murray F, Tate R, MacLean MR (2007) Molecular determinants in pulmonary hypertension: the role of PDE5. CRC Press, Boca Raton
- Rabe KF, Tenor H, Dent G, Schudt C, Nakashima M, Magnussen H (1994) Identification of PDE isozymes in human pulmonary artery and effect of selective PDE inhibitors. *Am J Physiol* 266:L536–L543
- Raymond DR, Wilson LS, Carter RL, Maurice DH (2007) Numerous distinct PKA-, or EPAC-based, signalling complexes allow selective phosphodiesterase 3 and phosphodiesterase 4 coordination of cell adhesion. *Cell Signal* 19:2507–2518
- Rybalkin SD, Bornfeldt KE (1999) Cyclic nucleotide phosphodiesterases and human arterial smooth muscle cell proliferation. *Thromb Haemost* 82:424–434
- Rybalkin SD, Bornfeldt KE, Sonnenburg WK, Rybalkina IG, Kwak KS, Hanson K, Krebs EG, Beavo JA (1997) Calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1C) is induced in human arterial smooth muscle cells of the synthetic, proliferative phenotype. *J Clin Invest* 100:2611–2621
- Rybalkin SD, Rybalkina I, Beavo JA, Bornfeldt KE (2002) Cyclic nucleotide phosphodiesterase 1C promotes human arterial smooth muscle cell proliferation. *Circ Res* 90:151–157
- Rybalkina IG, Tang XB, Rybalkin SD (2010) Multiple affinity states of cGMP-specific phosphodiesterase for sildenafil inhibition defined by cGMP-dependent and cGMP-independent mechanisms. *Mol Pharmacol* 77:670–677
- Schafer S, Ellinghaus P, Janssen W, Kramer F, Lustig K, Milting H, Kast R, Klein M (2009) Chronic inhibition of phosphodiesterase 5 does not prevent pressure-overload-induced right-ventricular remodelling. *Cardiovasc Res* 82:30–39
- Schermuly RT, Roehl A, Weissmann N, Ghofrani HA, Leuchte H, Grimminger F, Seeger W, Walmrath D (2001) Combination of nonspecific PDE inhibitors with inhaled prostacyclin in experimental pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 281:L1361–L1368
- Schermuly RT, Kreisselmeier KP, Ghofrani HA, Samidurai A, Pullamsetti S, Weissmann N, Schudt C, Ermert L, Seeger W, Grimminger F (2004) Antiremodeling effects of iloprost and the dual-selective phosphodiesterase 3/4 inhibitor tolafentrine in chronic experimental pulmonary hypertension. *Circ Res* 94:1101–1108
- Schermuly RT, Inholte C, Ghofrani HA, Gall H, Weissmann N, Weidenbach A, Seeger W, Grimminger F (2005) Lung vasodilatory response to inhaled iloprost in experimental pulmonary hypertension: amplification by different type phosphodiesterase inhibitors. *Respir Res* 6:76
- Schermuly RT, Pullamsetti SS, Kwapiszewska G, Dumitrascu R, Tian X, Weissmann N, Ghofrani HA, Kaulen C, Dunkern T, Schudt C, Voswinckel R, Zhou J, Samidurai A, Klepetko W, Paddenberger R, Kummer W, Seeger W, Grimminger F (2007) Phosphodiesterase 1 upregulation in pulmonary arterial hypertension: target for reverse-remodeling therapy. *Circulation* 115:2331–2339

- Selige J, Tenor H, Hatzelmann A, Dunkern T (2010) Cytokine-dependent balance of mitogenic effects in primary human lung fibroblasts related to cyclic AMP signaling and phosphodiesterase 4 inhibition. *J Cell Physiol* 223(2):317–326
- Simonneau G, Robbins IM, Beghetti M, Channick RN, Delcroix M, Denton CP, Elliott CG, Gaine SP, Gladwin MT, Jing ZC, Krowka MJ, Langleben D, Nakanishi N, Souza R (2009) Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 54:S43–S54
- Souness JE, Aldous D, Sargent C (2000) Immunosuppressive and anti-inflammatory effects of cyclic AMP phosphodiesterase (PDE) type 4 inhibitors. *Immunopharmacology* 47:127–162
- Stavros F, Kramer WG, Wilkins MR (2010) The effects of sitaxentan on sildenafil pharmacokinetics and pharmacodynamics in healthy subjects. *Br J Clin Pharmacol* 69:23–26
- Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF (2009) Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol* 297:L1013–L1032
- Sun CK, Lee FY, Sheu JJ, Yuen CM, Chua S, Chung SY, Chai HT, Chen YT, Kao YH, Chang LT, Yip HK (2009a) Early combined treatment with cilostazol and bone marrow-derived endothelial progenitor cells markedly attenuates pulmonary arterial hypertension in rats. *J Pharmacol Exp Ther* 330:718–726
- Sun XZ, Li ZF, Liu Y, Fang P, Li MX (2010) Inhibition of cGMP phosphodiesterase 5 suppresses MMP2 production in pulmonary artery smooth muscles cells. *Clin Exp Pharmacol Physiol* 37(3):362–367
- Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, Bedja D, Gabrielson KL, Wang Y, Kass DA (2005) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* 11:214–222
- Takimoto E, Koitabashi N, Hsu S, Ketner EA, Zhang M, Nagayama T, Bedja D, Gabrielson KL, Blanton R, Siderovski DP, Mendelsohn ME, Kass DA (2009) Regulator of G protein signaling 2 mediates cardiac compensation to pressure overload and antihypertrophic effects of PDE5 inhibition in mice. *J Clin Invest* 119:408–420
- Thompson WJ, Brooker G, Appleman MM (1974) Assay of cyclic nucleotide phosphodiesterases with radioactive substrates. *Methods Enzymol* 38:205–212
- Thompson PD, Zimet R, Forbes WP, Zhang P (2002) Meta-analysis of results from eight randomized, placebo-controlled trials on the effect of cilostazol on patients with intermittent claudication. *Am J Cardiol* 90:1314–1319
- Toque HA, Teixeira CE, Priviero FB, Morganti RP, Antunes E, De Nucci G (2008) Vardenafil, but not sildenafil or tadalafil, has calcium-channel blocking activity in rabbit isolated pulmonary artery and human washed platelets. *Br J Pharmacol* 154:787–796
- Trembath RC (2001) Mutations in the TGF-beta type 1 receptor, ALK1, in combined primary pulmonary hypertension and hereditary haemorrhagic telangiectasia, implies pathway specificity. *J Heart Lung Transplant* 20:175
- Truss MC, Stief CG, Uckert S, Becker AJ, Wefer J, Schultheiss D, Jonas U (2001) Phosphodiesterase 1 inhibition in the treatment of lower urinary tract dysfunction: from bench to bedside. *World J Urol* 19:344–350
- Tsai BM, Turrentine MW, Sheridan BC, Wang M, Fiore AC, Brown JW, Meldrum DR (2006) Differential effects of phosphodiesterase-5 inhibitors on hypoxic pulmonary vasoconstriction and pulmonary artery cytokine expression. *Ann Thorac Surg* 81:272–278
- Tuder RM, Groves B, Badesch DB, Voelkel NF (1994) Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 144:275–285
- Tuder RM, Abman SH, Braun T, Capron F, Stevens T, Thistlethwaite PA, Haworth SG (2009) Development and pathology of pulmonary hypertension. *J Am Coll Cardiol* 54:S3–S9
- Wagner RS, Smith CJ, Taylor AM, Rhoades RA (1997) Phosphodiesterase inhibition improves agonist-induced relaxation of hypertensive pulmonary arteries. *J Pharmacol Exp Ther* 282:1650–1657

- Wang C, Li JF, Zhao L, Liu J, Wan J, Wang YX, Wang J (2009) Inhibition of SOC/Ca²⁺/NFAT pathway is involved in the anti-proliferative effect of sildenafil on pulmonary artery smooth muscle cells. *Respir Res* 10:123
- Wharton J, Strange JW, Moller GM, Growcott EJ, Ren X, Franklyn AP, Phillips SC, Wilkins MR (2005) Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells. *Am J Respir Crit Care Med* 172:105–113
- Wilkens H, Guth A, Konig J, Forestier N, Cremers B, Hennen B, Bohm M, Sybrecht GW (2001) Effect of inhaled iloprost plus oral sildenafil in patients with primary pulmonary hypertension. *Circulation* 104:1218–1222
- Yan C, Miller CL, Abe J (2007) Regulation of phosphodiesterase 3 and inducible cAMP early repressor in the heart. *Circ Res* 100:489–501
- Zaccolo M, Movsesian MA (2007) cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res* 100:1569–1578
- Zhang L, Murray F, Zahno A, Kanter JR, Chou D, Suda R, Fenlon M, Rassenti L, Cottam H, Kipps TJ, Insel PA (2008) Cyclic nucleotide phosphodiesterase profiling reveals increased expression of phosphodiesterase 7B in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 105:19532–19537
- Zhao L, Mason NA, Morrell NW, Kojonazarov B, Sadykov A, Maripov A, Mirrakhimov MM, Aldashev A, Wilkins MR (2001) Sildenafil inhibits hypoxia-induced pulmonary hypertension. *Circulation* 104:424–428
- Zhu S, White RE, Barman SA (2008) Role of phosphodiesterases in modulation of BKCa channels in hypertensive pulmonary arterial smooth muscle. *Ther Adv Respir Dis* 2:119–127

Treatment of Erectile Dysfunction and Lower Urinary Tract Symptoms by Phosphodiesterase Inhibitors

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Abstract To date, it is widely accepted that several disorders of the male and female urogenital tract, such as erectile dysfunction, bladder overactivity, urinary stone disease, the benign prostatic syndrome, as well as symptoms of female sexual arousal and orgasmic dysfunctions, can be therapeutically approached by influencing the function of the smooth musculature of the respective organs. To achieve a pronounced drug effect without significant adverse events, a certain degree of tissue selectivity is mandatory. Selective intervention in intracellular pathways regulating

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smooth muscle tone has become the most promising strategy to modulate tissue and organ function. Since the concept of taking a pill to relieve symptoms of lower urinary tract dysfunction is now widely accepted following the successes of phosphodiesterase 5 (PDE5) inhibitor treatment of erectile dysfunction, the treatment of urological diseases has focused on orally available drugs acting via influencing intracellular signaling pathways, thereby combining a high response rate with the advantage of an on-demand intake. Specifically, the use of isoenzyme-selective PDE inhibitors offers great opportunities in the medical treatment of various genitourinary diseases. These agents are regarded to be safe and to be efficacious, i.e., having a fast onset of drug action and an improved effect-to-side-effect ratio. As experience with this class of compounds and their use in urology is rapidly growing, basic and clinical research in this field will most likely expand the pharmacological armamentarium of innovative treatment options in the next few years. The purpose of this review is to summarize current, as well as potential, upcoming indications for the use of PDE inhibitors in the pharmacotherapy of male erectile dysfunction and lower urinary tract symptoms.

Keywords Lower urinary tract symptoms · Male erectile dysfunction · Oral pharmacotherapy · Phosphodiesterase (PDE) inhibitors

1 Introduction

Cyclic nucleotide monophosphates cyclic AMP (cAMP) and cyclic GMP (cGMP) are important intracellular mediators of several processes, including smooth muscle motility, electrolyte homeostasis, neuroendocrine signals, and retinal phototransduction (Antoni 2000; Lucas et al. 2000). Following a physiological signal, e.g., the release of nitric oxide (NO) from nonadrenergic, noncholinergic nerve terminals or activation of specific G-protein-coupled receptors on the outer cell surface, cyclic nucleotides are synthesized from their corresponding nucleoside triphosphate by the activity of adenylyl and guanylyl cyclases. This increase in cAMP or cGMP triggers a signal transduction cascade, which encompasses the activation of cyclic nucleotide-dependent protein kinases (cAK, cGK), subsequent phosphorylation of the actin-myosin system, as well as Ca^{2+} channels and ATP-driven Ca^{2+} pumps located in the outer cell membrane or the membrane of the sarcoplasmic reticulum. This cascade leads to a reduction in cytosolic Ca^{2+} and, finally, to smooth muscle relaxation. Cyclic nucleotides are degraded by phosphodiesterase (PDE) isoenzymes, a heterogeneous group of hydrolytic enzymes. It is because of their central role in smooth muscle tone regulation that PDEs have become an attractive target for drug development. PDEs are classified according to their preference or affinity for cAMP or/and cGMP, kinetic parameters of cyclic nucleotide hydrolysis, relative sensitivity to inhibition by various compounds, allosteric regulation by other molecules, amino acid sequences, and chromatographic behavior on anion exchange columns.

Eleven families of PDE isoenzymes can be distinguished: Ca^{2+} /calmodulin-stimulated PDE (PDE1), cGMP-stimulated PDE (PDE2), cGMP-inhibited PDE (PDE3), cAMP-specific PDE (PDE4), cGMP-binding, cGMP-specific PDE (PDE5), and the cGMP-binding, cGMP-specific PDE of mammalian rods and cones (PDE6). While PDE7 (which has high affinity and specificity for cAMP) and PDE8 (IBMX-insensitive) have preferred selectivity for cAMP, PDE9 exclusively degrades cGMP. PDEs 10 and 11 can inactivate both cAMP and cGMP (Michaeli et al. 1994; Han et al. 1997; Fisher et al. 1998a; Fisher et al. 1998b; Loughney et al. 1999; Fawcett et al. 2000). Some of these isoenzyme families consist of more than one gene (isogenes) and some genes are alternatively spliced so that, to date, more than 50 isoenzymes or variants have been described (Conti and Jin 1999; Essayan 2001). Some PDE genes are also variably expressed in different tissues. For example, PDE2 is predominantly found in vascular smooth muscle. Expression of PDE5 in the corpus cavernosum and the cGMP-mediated relaxation of the cavernous smooth muscle during sexual stimuli have made inhibition of this enzyme a clinical benefit in the management of erectile dysfunction (ED). PDE7 is abundant in skeletal muscle and is also present in the human kidney. Although expressed in other tissues, high levels of PDE8, -10, and -11 are found in the testis, and PDE9 is expressed in intestinal smooth muscle, skeletal (striated) muscle, and brain (Rybulkin et al. 2003; Maurice et al. 2003). To date, 6 out of these 11 isoenzymes (PDE1, -2, -3, -4, -5, and -11) have been proven to be of pharmacological importance. Since the distribution and functional significance of PDE isoenzymes can vary in different tissues, isoenzyme-selective inhibitors have the potential to exert specific effects on the target tissue.

2 PDE5 Inhibitors in Male Erectile Dysfunction

Due to the demographic development in the populations of industrialized countries, the prevalence of ED has dramatically increased. While, in 1995, ~152 million men worldwide reported that they suffered from ED, in 2005, it has been estimated that 320 million men will report this as a medical problem by the year 2025. Since the early 1980s, the mechanisms of erectile physiology and pathophysiology have been clarified in detail and extensive coverage was made on the nitric oxide (NO)-cGMP pathway and its role in the initiation and maintenance of penile erection (Holmquist et al. 1991; Rajfer et al. 1992). The discovery of NO and cGMP as the major effectors in penile smooth muscle relaxation was a landmark and has led to the identification of certain drugs that can foster elevation of intracellular levels of cGMP. Among these agents are the nitric oxide donor drugs such as sodium nitroprusside, nitroglycerin and linsidomine (SIN-1), and selective inhibitors of PDE5 (Truss et al. 1994; Heaton and Morales 1990; Ückert et al. 2001a). Especially, the use of selective PDE5 inhibitors in the treatment of ED has gained widespread acceptance in the field of urology and persists as an important topic. Sildenafil, vardenafil, and tadalafil are nonhydrolysable analogs of cGMP that

counteract the degradation of this cyclic nucleotide by binding to the catalytic site of PDE5 and blocking access by the substrate, cGMP. Hereby, enhancement of NO-initiated relaxations of cavernous erectile tissue can be obtained (Kalsi and Kell 2004). The efficacy of the PDE5 inhibitors has been evaluated in myriad clinical trials involving thousands of male subjects with different causes of impotence: psychogenic, with no identifiable organic cause, as well as patients with diabetes and histories of spinal cord injury and pelvic surgery (radical prostatectomy). In all trials, men receiving a PDE5 inhibitor more often reported erections sufficient for sexual intercourse than did those who received placebo (Fink et al. 2002; Stief et al. 2004; Eardly and Cartledge 2002). However, the action of PDE5 inhibitors requires some degree of neuronal input to the corpus cavernosum as well as the intact cavernous endothelial structures. The most common side effects that are reported following the use of PDE5 inhibitors include headache (18%), flushing (11%), dyspepsia (7%), nasal congestion (5%) and are related to the presence of PDE5 in tissues other than the corpus cavernosum (Küthe et al. 2000). With regard to the cardiovascular safety, it has been shown that PDE5 inhibitors do not affect the ability of patients with coronary artery disease to maintain a level of exercise similar to that required for sexual activity and that patients do not experience significant side effects from the use of these medications. Studies to evaluate the interactions between PDE5 inhibitors and organic nitrates demonstrated only modest synergistic effects on the nitrate-induced reduction in mean systolic and diastolic blood pressure (Mazzu et al. 2001; Thadani and Mazzu 2002; Emmick et al. 2002). Nevertheless, due to their mechanisms of action, that is the enhancement of cGMP accumulation due to increased synthesis and decreased cGMP breakdown, the use of PDE5 inhibitors is contraindicated in ED patients taking nitrates.

3 Treating ED by Combining PDE5 Inhibitors with Other Drugs

Since several classes of drugs with different modes of pharmacological action demonstrate efficacy in treating ED, this creates the potential for therapeutic combination of some of these drugs. Currently, only the combined application of sildenafil and alprostadil (PGE1) has been studied comprehensively despite other possibilities. In a placebo-controlled cross-over study, 40 patients with ED, who had experienced unsatisfactory erections with both 50 and 100 mg of sildenafil, were treated for 4 weeks with intracavernous injections of PGE1 (20 µg) (two per week given in the clinic and then provided with either placebo or 50 mg sildenafil for use at home). In a subset of 26 subjects (65%), the IIEF-Erectile Function domain score, the main outcome measure, was found to be considerably higher with the combined PGE1/sildenafil (50 mg) treatment than with PGE1/placebo or sildenafil (50 or 100 mg) alone (Gutierrez et al. 2005). There is also evidence that the combination of an orally effective PDE5 inhibitor, such as sildenafil, tadalafil,

or vardenafil, with testosterone replacement therapy might be effective in hypogonadal patients with ED when testosterone alone fails (or vice versa). Greco et al. (2006), by means of an extensive MEDLINE search (2003–2006), reviewed relevant data from basic and clinical studies regarding the efficacy of combined testosterone replacement and PDE5 inhibitor therapy. Their analysis revealed that, due to the molecular mechanisms of action of androgen hormones and its receptor in the regulation of PDE5 expression in the human corpus cavernosum, PDE5 inhibitors might be ineffective in hypogonadal men with ED. Thus, the efficacy of this class of drugs might be enhanced by concomitant testosterone administration whenever necessary. They concluded that screening for hypogonadism in all men with ED is necessary to identify men with severe or moderate hypogonadism, those who may be refractory to PDE5 inhibitor treatment and who may benefit from testosterone supplementation (Greco et al. 2006). These considerations may improve the clinical management of patients who are unresponsive to treatments with PDE5-selective inhibitors. The combination of centrally acting agents, such as the dopaminergic agonist apomorphine, with PDE5 inhibitors has also been considered to be an attractive approach because the two therapies target different signaling mechanisms (Sommer and Engelmann 2004). However, no prospective randomized clinical trials with adequate numbers of patients have yet been conducted to provide a scientific rationale for this combination strategy.

4 Upcoming PDE5 Inhibitors

4.1 *Udenafil (DA-8159)*

Udenafil is an oral active PDE5 inhibitor currently available in South Korea for the treatment of ED. Phase II clinical data demonstrated that in men with mild-to-severe ED, the drug produced a significant improvement in erectile function after 12 weeks of treatment. Udenafil has been reported as being well tolerated. In a rat model, the effects of acute treatment with DA-8159 were investigated on erectile dysfunction associated with hypercholesterolemia or diabetes. In the animals, the administration of DA-8159 (0.3 or 1 mg/kg) induced a dose- and frequency-dependent increase in intracavernous pressure (ICP). Chronic treatment with DA-8159 (20 mg/kg/day) over 5 months restored the erectile responses induced by electric stimulation, improved endothelial function as assessed by recording thoracic aorta relaxation *in vitro* in response to the cumulative addition of acetylcholine to the bathing medium, and also significantly decreased plasma levels of endothelin and asymmetrical dimethyl arginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS) activity (Kang et al. 2005). These results provided a rationale for the potential use of DA-8159 for treating ED secondary to hypercholesterolemia.

4.2 *Avanafil*

VIVUS Inc. (Mountain View, CA, USA) is in the process of developing avanafil, a fast-acting, highly selective PDE5 inhibitor, as an oral medication for the treatment of ED. VIVUS has announced favorable data from a phase II at-home, multicenter, double-blind, randomized, and parallel-design study. Patients were instructed to attempt sexual intercourse 30 min after taking avanafil, with no food or alcohol restrictions. Results showed that up to 84% of avanafil doses resulted in erections sufficient for vaginal penetration, as compared with placebo. No serious adverse events were reported. Positive data were also demonstrated in a twice-daily study and nitrate interaction study. The results from the twice-daily study showed no significant plasma accumulation of the drug after the twice-a-day treatment regimen. Results from the nitrate study revealed that avanafil had only little impact on blood pressure and heart rate (Gur et al. 2008; Hatzimouratidis and Hatzichristou 2008). In comparison with sildenafil, vardenafil, and tadalafil, avanafil presents a unique chemical structure differing from the standard (nucleo)base/sugar/phosphate diester model of many PDE5 inhibitors. In the avanafil molecule, the central structure is formed by a nitrogen derivative of a pyrimidine carboxamide where the nitrogen atom of the amide is bound to a pyrimidinylmethyl group. It seems likely that the ribose-phosphate component is represented by a cyclic chloromethoxy benzylamino structure where the Cl-atom and the methoxy-ligand resemble the phosphate group. Thus, theoretically, the inhibitor can bind to the catalytic site of PDE5 regardless of the spatial orientation of the molecule. This property may significantly increase the affinity of the inhibitor toward PDE5 and, thus, the clinical effectiveness of the drug.

4.3 *Lodenafil*

Lodenafil carbonate is a novel PDE5 inhibitor that was recently introduced into the clinical phase of development. The effects of lodenafil have been investigated *in vitro* on the relaxation of penile erectile tissue and the activity of PDE5 in crude extracts from human platelets. The drug caused concentration-dependent relaxation of both rabbit and human corpus cavernosum and significantly amplified the nitric oxide-dependent relaxation of the cavernous tissue evoked by acetylcholine or transmural electrical field stimulation (EFS). No effects of the drug were noted on mean arterial pressure. Lodenafil has been shown to be approximately twofold more potent than sildenafil for inhibition of the hydrolysis of cGMP in crude extracts prepared from human platelets (Toque et al. 2008). In a phase II, prospective, randomized, double-blind and placebo-controlled trial in a cohort of 72 men presenting with ED, lodenafil carbonate (20, 40 or 80 mg) significantly improved IIEF erectile domain scores and sexual encounter profile (SEP) scores of the patients. The drug was well tolerated, adverse reactions were mild and

included headache, rhinitis, flushing, color visual disorders, and dyspepsia. On the basis of these observations, lodenafil is considered as an attractive agent to be used in the immediate future in the pharmacotherapy of ED (Glina et al. 2009).

5 PDE Inhibitors in the Treatment of ED: Beyond PDE5

Prior to the generally accepted clinical use of PDE5 inhibitors for the management of ED, compounds proceeding through cAMP-dependent mechanisms were historically widely used in ED self-injecting regimes. Related drugs include prostaglandin E1 (PGE1), vasoactive intestinal polypeptide and forskolin, a diterpene-stimulating adenylyl cyclase (AC) (Ottensen et al. 1984; Porst 1996; Cahn et al. 1997). By binding to specific G-protein-coupled receptors in the membrane of smooth muscle cells, prostaglandins or peptidergic ligands can activate AC with increased intracellular production of cAMP. Cyclic AMP preferably acts on the cAMP-dependent protein kinase (cAK), which antagonizes the cellular contractile mechanism via modulation of the activities of other proteins, e.g., Ca^{2+} -channels, but it can also cross-activate the cGMP-dependent protein kinase (cGK) to affect many of the same processes. The intracellular level of cAMP in human erectile tissue is mainly regulated by cAMP production by adenylyl cyclases and cAMP-degrading PDEs such as PDE3 and PDE4. Although activation of the AC/cAMP signaling system is established as an effective relaxation-producing pathway in human cavernous tissue, only few studies have been conducted to characterize key proteins of the AC/cAMP axis. Evidence for the presence of PDE3 and PDE4 in human erectile tissue has been shown and messenger RNA transcripts encoding for PDE3A, PDE4(A–D), PDE7A, and PDE8A, all of which are known to hydrolyse cAMP, were shown by means of RT-PCR and Northern Blot analysis (Rajasekaran et al. 1998; K uthe et al. 2001). Results obtained *in vitro* suggest that PDE3 and PDE4 might be the predominant cAMP-hydrolyzing isoenzymes in human corpus cavernosum (Taher et al. 1997; Stief et al. 1998). Interestingly, it has been shown that the relaxation of α_1 -adrenoceptor-mediated tension of isolated human penile erectile tissue induced by sildenafil and tadalafil was reversed by the protein kinase A inhibitor Rp-8-CPT-cAMPS, suggesting an involvement of cAMP-mediated mechanisms in the action of PDE5 inhibitors. A significant role of PDE4 and cAMP in the control of human erectile tissues is further supported by the finding that immunoreactions specific for PDE4 and PDE4A were detected in cavernous endothelial and neuronal structures ( uckert et al. 2004). On the basis of these observations, an important complementary role might be considered for the AC/cAMP/cAK pathway in the regulation of cavernous smooth muscle tone. This provides a rationale to further investigate the effects of selective PDE4-inhibitors, as well as compounds known to inhibit both PDE5 and PDE4, in models for erectile function and dysfunction (Guay et al. 2002; Silver et al. 1997).

6 Nonmalignant Diseases of the Prostate: The Benign Prostatic Syndrome

Benign prostatic syndrome (BPS) represents a major health care problem in western countries. BPS comprises obstructive and irritative symptoms: lower urinary tract symptomatology (LUTS), as well as benign prostatic enlargement (BPE) with variable degrees of bladder outlet obstruction (BOO) (Guess 1995; Chiricos and Sanford 1996). Major symptoms include urinary frequency, nocturia, and slow stream. It is estimated that approximately 50% of men older than 50 years have moderate to severe symptoms arising from LUTS (Jacobsen et al. 1993). The current pharmacological management of LUTS and BPE involves alpha₁-adrenergic blockers, such as alfuzosin, doxazosin, and tamsulosin, to diminish urethral resistance by reducing the tension of smooth muscle fibers located in the transitional zone and periurethral region (Hieble and Ruffolo 1996). Intervention into the hormonal control of prostate growth by using inhibitors of 5-alpha-reductase activity is another approach to ease symptoms (Andersen et al. 1995; Roehrborn et al. 2004).

The expression of several cAMP- and cGMP-PDE isoenzymes (PDEs 1, 2, 4, 5, 7, 8, 9, 10) in the human prostate was shown by means of molecular biology and protein chemistry. Later, the distribution of PDE4 and PDE5 in stromal and glandular areas of the transition zone was shown using an immunohistochemical approach (Ückert et al. 2001b; Ückert et al. 2006). In organ bath studies, the tension of prostate strip preparations mediated via the activation of alpha₁-adrenergic receptors was dose-dependently reversed by the PDE4 inhibitor rolipram and PDE5 inhibitor sildenafil (Ückert et al. 2001b). The role of PDE5 inhibitors in the treatment of symptoms of LUTS/BPH has been addressed by some open-label studies and, more recently, placebo-controlled clinical trials. Sairam et al. (2002) were the first who examined the effects of sildenafil citrate in patients presenting with ED and LUTS. From the 112 patients enrolled in the open-label study, 20 subjects complained of LUTS, from these, 32% had moderate-to-severe symptoms (IPSS > 7). After 12 weeks of treatment with sildenafil, there was an overall improvement in the IPSS and LUTS-specific quality of life (QoL) score. All patients who had severe LUTS showed a moderate improvement of the disease, 60% of those who initially presented with moderate LUTS showed only a mild symptomatology. The authors concluded that treatment with sildenafil appears to improve urinary symptom scores (Sairam et al. 2002). McVary et al. (2007a, b) assessed in a randomized, double-blind, placebo-controlled trial the effects of sildenafil given for 12 weeks in 366 men suffering from ED and LUTS secondary to BPH (IPSS ≥ 12). Patients received 50 mg sildenafil at bed-time or up to 1 h before sexual activity for at least 2 weeks, followed by 100 mg sildenafil once daily for 10 weeks. Primary outcome measures were changes in the erectile function domain of the IIEF, secondary outcome measures were changes in all other domains of the IIEF and IPSS including QoL, BPH *Impact Index* (BPH II), and Q_{max}. Sildenafil significantly improved LUTS, mean IPSS decreased by 6.3 points vs. 1.93 in the placebo group. Interestingly, patients with severe LUTS

(-8.6 ± -2.4) experienced greater improvement in IPSS than those with moderate LUTS (-3.6 ± -1.7). A significant reduction of bladder storage symptoms and an increase in the BPH II and QoL were also noted in the treatment arm while no significant changes in Q_{\max} were registered. From the later findings, the authors concluded that extraprostatic pathophysiological mechanisms related to an impairment of the activity of the NO-system might be involved in the etiology of LUTS (McVary et al. 2007a). The efficacy and safety of tadalafil (CIALISTM) were also investigated in a randomized, double-blind, placebo-controlled study in men with moderate-to-severe LUTS secondary to BPH. Following a 4-week placebo run-in phase, 281 men were randomized to 5 mg tadalafil for 6 weeks, followed by dose escalation to 20 mg for another 6 weeks or placebo for 12 weeks. Primary end point was the change in IPSS after 6 and 12 weeks of treatment. Secondary efficacy endpoints included patient IPSS, QoL, BPH II, and the LUTS *global assessment questionnaire* (GAQ), as well as parameters from uroflowmetric measurements (Q_{\max}). After 6 weeks and 12 weeks, tadalafil showed significant improvement in those with LUTS, the respective mean change from baseline in IPSS was -2.8 in tadalafil group vs. -1.2 in the placebo group (6 weeks) and -3.8 (verum) vs. -1.7 (placebo). Mean subscores related to irritative and obstructive symptoms also significantly improved in patients who had received tadalafil. Except for BPH II after 6 weeks, all assessments of the disease-related QoL had significantly improved after treatment with tadalafil. In contrast, no significant changes in uroflowmetric values were observed. (McVary et al. 2007b). In an 8-week randomized, double-blind, placebo-controlled, multicenter study, Stief et al. (2008) examined the efficacy of vardenafil (LEVITRATM) in a cohort of 222 men (aged 45–64 years) presenting with LUTS/BPH (IPSS ≥ 12). Patients were randomized either to vardenafil (10 mg twice daily) or placebo. Efficacy outcome included changes in peak urinary flow rate, as well as scores from the IPSS and a nine-item BPH-specific QoL questionnaire (UROLIFE QoL 9). After treatment, a decrease of 5.9 points in the IPSS was observed in the vardenafil group vs. 3.6 points in the placebo group. Significant changes in the IPSS subscores for storage and voiding symptoms and an improvement in the UROLIFE QoL were also noted in the vardenafil group. Since baseline values were already close to normal, Q_{\max} and postvoiding residual urine volume did not change significantly with treatment (Stief et al. 2008). It was shown more recently that sildenafil treatment also improves Q_{\max} and Q_{ave} (mean average flow rate) rates in men with LUTS suggestive of BPE. A single dose of the PDE5-inhibitor (100 mg) resulted in an improvement in Q_{\max} in 29 of 38 patients. Q_{ave} and the mean voided volumes of the patients also increased while no significant differences were registered in the Q_{\max} , Q_{ave} , and voided volumes of the control group before and after placebo (Gülcer et al. 2008). These results support the use of PDE5 inhibitors for treating LUTS and urinary obstruction secondary to BPE. The combination of the PDE5 inhibitors sildenafil (25 mg once daily) or tadalafil (10 mg once daily) and the alpha-adrenoceptor antagonist alfuzosin (10 mg once daily) to treat LUTS has also been evaluated. Men with previously untreated LUTS (and ED) were randomized to either alfuzosin, sildenafil, or tadalafil or the combination of the alpha-blocker and a PDE5 inhibitor

for 12 weeks. Primary endpoints with regard to the LUT symptomatology were changes from baseline in IPSS, maximum urinary flow rate (Q_{\max}), medium urinary flow rate (Q_{ave}), and postvoid residual urine (PVRU) volume. Improvement in IPSS was significant in all treatments arms, but most pronounced with the drug combinations. PVRU, Q_{\max} , frequency, and nocturia were significantly improved with alfuzosin only and the combination regimen. From the results, demonstrating the significant improvement in uroflowmetry measures and IPSS, it was concluded that combined therapy is more effective than monotherapy with either agent to improve voiding dysfunction in men with LUTS suggestive of BPH (Kaplan et al. 2007; Liguori et al. 2009). Combining a PDE5 inhibitor with a 5-alpha-reductase inhibitor (5-ARI, such as dutasteride or finasteride) or an antimuscarinic drug (such as darifenacin, fesoterodine, oxybutinin, or tolterodine) has also been suggested as a potential treatment for LUTS. However, to date, no clinical evidence has been presented indicating that such treatment regimens are effective and safe in the individual patient (Andersson 2008).

7 Bladder Overactivity

Anticholinergic drugs are currently the therapy of choice to treat urgency and urge incontinence (Andersson et al. 2001). Nevertheless, to date, muscarinic receptor blockers that act exclusively on detrusor smooth muscle are not available. Moreover, the unstable detrusor seems to be regulated in part by noncholinergic mechanisms. These factors may explain the common side effects and limited clinical efficacy of anticholinergic medications. The specific modulation of intracellular second messenger pathways may offer a promising possibility to achieve selective modulation of tissue function, especially with regard to the contraction and relaxation of human urinary bladder smooth musculature. Using chromatographic methods, Truss et al. (1996a) were the first who reported the spectrum of PDEs in the human detrusor; these include PDE1 (cAMP/cGMP-PDE, Ca^{2+} /calmodulin-dependent), PDE2 (cAMP-PDE, cGMP-dependent), PDE3 (cAMP-PDE, cGMP-inhibited), PDE4 (cAMP-PDE), and PDE5 (cGMP-PDE). They also demonstrated relaxant responses of isolated human detrusor strip preparations contracted by the muscarinic agonist carbachol to the nonspecific PDE inhibitor papaverine and the PDE1 inhibitor vinpocetine. The relaxing effects of the drugs were paralleled by an increase in tissue levels of cAMP and cGMP. They concluded from their findings that the cAMP-pathway and PDE1 might be of functional significance in the control of detrusor smooth muscle contractility (Truss et al. 1996b). The predominant expression of PDE1 in the human detrusor was later confirmed by RT-PCR analysis (Ückert et al. 2009). Results from a randomized, double-blind, placebo-controlled study to assess effects of the PDE1 inhibitor vinpocetine in patients with urgency and urge incontinence, who had failed standard pharmacological therapy, demonstrated that vinpocetine was superior to placebo with regard to the clinical outcome parameters micturition frequency, bladder volume at first sensation, bladder

volume at voiding desire, maximum detrusor pressure, and voided volume (Truss et al. 2001). The hypothesis that detrusor smooth muscle relaxation is mainly mediated by the cAMP pathway was later supported by a study by Oger et al. (2007), who aimed to determine the effect of rolipram, a PDE4 inhibitor, on carbachol-induced contractions of human detrusor in vitro. They found that rolipram (1 nM–30 μ M) exerted a moderate relaxing effect on the tension induced by carbachol, but inhibited more effectively the phasic contractile activity of the tissue. The effect of rolipram on the tonic contraction was enhanced in strips that had not been denuded of the urothelium or when the production of cAMP was increased by the adenylyl cyclase activator forskolin. The authors concluded that PDE4 is involved in the control of the phasic myogenic activity of human bladder and suggested that PDE4 inhibitors might represent an attractive strategy for treatment of OAB (Oger et al. 2007).

Recently, the efficacy of the PDE5 inhibitor vardenafil was also assessed in a single center, randomized, double-blind, placebo-controlled trial in a group of 25 male patients with spinal cord injuries and with micturition disorders who were on oxybutynin treatment. Following a baseline urodynamic testing, a second test was performed 1–3 h after administration of 20 mg vardenafil or placebo. Primary endpoints were changes in maximum detrusor pressure during voiding, maximum cystometric capacity, and detrusor volume at first (overactivity) sensation. Although NO does not appear to have direct smooth muscle regulatory functions in the detrusor muscle, vardenafil administration significantly decreased maximum detrusor pressure, considerably improved cystometric capacity, and increased volume at first sensation (Gacci et al. 2007).

Thus, modulating the activity of PDE isoenzymes might represent a novel approach that could avoid the limitations of anticholinergic therapy in patients with lower urinary tract dysfunction. Future studies will delineate as to whether PDE inhibitors, such as the PDE1 inhibitor vinpocetine or selective PDE5 inhibitors, may have significance in the treatment of detrusor instabilities and urge incontinence.

8 Urinary Stone Disease (“Ureteral Colic”)

Very much in contrast to the urinary bladder, prostate or urethra, little is known on the peripheral neurotransmission responsible for the relaxation of ureteral smooth muscle. Research on ureteral neurotransmitters focused on afferent innervation; studies on the efferent limb of the autonomic innervation of the ureter were done over two decades ago when the concept of nonadrenergic/noncholinergic innervation was not yet established. From 1990 to 1999, reports on the localization of VIP and NOS in nerves supplying the ureter were published, but no in vitro studies were done to corroborate the findings (Smet et al. 1994). Later, it was demonstrated that the tension of isolated human ureteral tissue was dose-dependently reversed by the NO donor drug molsidomine (SIN-1); preincubation with the sGC-inhibitor methylene blue

significantly reduced the relaxation response. However, no effects of the NOS-inhibitor L-NORAG were observed on the relaxation induced by means of EFS. Immunohistochemistry revealed NOS-containing neuronal axons as well as nerve endings in the muscular layers of the ureter (Stief et al. 1996). Taher et al. (1994) reported the presence of the cGMP PDE type 5 (PDE5), cAMP/cGMP PDE1, and cAMP PDE2 and PDE4 in cytosolic supernatants prepared from human ureteral tissue. Using the organ bath technique, they demonstrated the potency of the PDE4 inhibitor rolipram and dual PDE5/PDE1 inhibitor zaprinast to reverse the tension induced by KCl of circular ureteral segments (Taher et al. 1994). Kühn et al. (2000) later confirmed the relaxing properties of inhibitors of PDE4 (rolipram) and PDE5 (E 4021, MSPP) on isolated human ureteral smooth musculature and showed that these effects were paralleled by an elevation in intracellular levels of cAMP or cGMP, respectively. Based on the results from the experiments on the effects of the NO-donor drugs sodium nitroprusside and SIN-1, and the PDE5 inhibitor zaprinast on the tension induced by KCl of proximal segments of the human ureter, Saighi et al. (2000) concluded that cGMP is an important second messenger in the signaling pathway leading to the relaxation of the ureteral smooth muscle. In contrast, Gratzke et al. (2007) registered marginal effects of PDE5 inhibitors (ildenafil, sildenafil, and tadalafil) on the tension induced by KCl of human ureteral tissue. Although the responses were paralleled by a three- to fourfold increase in tissue level of cGMP, the maximum relaxation (R_{\max}) induced by the drugs ranged from only 23 to 6.0% (Gratzke et al. 2007). In an in vivo rabbit model, Becker et al. (1998) examined the potential of the PDE4 inhibitor rolipram in comparison to nonselective PDE inhibitors (papaverine and theophylline) and the muscarinic antagonist scopolamine to induce ureteral relaxation. They found that only rolipram induced pronounced decreases in intraluminal (intraureteral) pressure as well as in the amplitude and frequency of the phasic (peristaltic) contractions of the ureter. No considerable effects on the systemic circulation were observed, whereas the application of scopolamine, papaverine, or theophylline exerted no or only short-lasting effects on the ureter but significantly affected systemic blood pressure of the animals (Becker et al. 1998). It was concluded from these findings that the application of PDE inhibitors, especially those of cAMP PDE4, seems promising to facilitate effectively and with minimal side effects the spontaneous passage of distal ureteral stones and relieve ureteral colic pain.

9 Conclusion

Based on the knowledge of the physiological mechanisms regulating the male and female genitourinary tract, the use of selective PDE inhibitors has been suggested to be a logical approach for the treatment of various urological diseases. The unending charge to conceive first-line treatments demonstrating advanced efficacy over previous options offers a promising future for the use of PDE inhibitors in the therapy of diseases of the urinary tract and reproductive tissues. Although some approaches are likely to involve the NO/cGMP-cascade, other strategies should also

take into account modulation of the cAMP-pathway, as well as the combination of active agents (e.g., combining a PDE5 inhibitor and a NO donor or NO-independent activator of the soluble guanylyl cyclase) to affect multiple peripheral intracellular targets. It is assumed that PDE inhibitors will be efficacious in terms of promoting normal organ function and exert limited systemic adverse events.

References

- Andersen JT, Ekman P, Wolf H, Beisland HO, Johansson JE, Kontturi M, Lehtonen T, Tveter K (1995) Can finasteride reverse the progress of benign prostatic hyperplasia? A two-year placebo-controlled study. *Urology* 46:631–637
- Andersson KE (2008) How many drugs for LUTS due to BPH are too many? *J Urol* 180: 811–812
- Andersson KE, Chapple C, Wein A (2001) The basis for drug treatment of the overactive bladder. *World J Urol* 19:294–298
- Antoni FA (2000) Molecular diversity of cAMP signaling. *Front Neuroendocrinol* 21:103–132
- Becker AJ, Stief CG, Meyer M, Truss MC, Forssmann WG, Jonas U (1998) The effect of the specific phosphodiesterase 4 inhibitor rolipram on the ureteral peristalsis in vitro and in vivo. *J Urol* 160:920–925
- Cahn D, Melman A, Valcic M, Christ GJ (1997) Forskolin: a promising new adjunct to intracavernous pharmacotherapy. *J Urol* 155:1789–1794
- Chiricos TN, Sanford E (1996) Cost consequences of surveillance, medical management or surgery for benign prostatic hyperplasia. *J Urol* 155:1311–1316
- Conti M, Jin SL (1999) The molecular biology of cyclic nucleotide phosphodiesterases. *Prog Nucleic Acid Res Mol Biol* 63:1–38
- Eardly I, Cartledge J (2002) Tadalafil (CIALIS) for men with erectile dysfunction. *Int J Clin Pract* 56:300–304
- Emmick JT, Stuewe SR, Mitchell M (2002) Overview of the cardiovascular effects of tadalafil. *Eur Heart J* 4(suppl H):H32–H47
- Essayan DM (2001) Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol* 108:671–680
- Fawcett L, Baxendale R, Stacey P, McGrouther C, Harrow I, Soderling S, Hetman J, Beavo JA, Philips SC (2000) Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proc Natl Acad Sci USA* 97:3702–3707
- Fink HA, McDonald R, Rutks IR, Nelson DB, Wilt TJ (2002) Sildenafil for male erectile dysfunction: a systematic review and meta-analysis. *Arch Intern Med* 162:1349–1360
- Fisher AD, Smith JF, Pillar JS, St Denis S, Cheng JB (1998a) Isolation and characterization of PDE8A, a novel cAMP-specific phosphodiesterase. *Biochem Biophys Res Commun* 246:570–577
- Fisher AD, Smith JF, Pillar JS, St Denis S, Cheng JB (1998b) Isolation and characterization of PDE9A, a novel cGMP-specific phosphodiesterase. *J Biol Chem* 273:15559–15564
- Gacci M, del Popolo G, Macchiarella A, Celso M, Vittori G, Lapini A, Serni S, Sandner P, Maggi M, Carini M (2007) Vardenafil improves urodynamic parameters in men with spinal cord injury: results from a single dose, pilot study. *J Urol* 178:2040–2043
- Glina S, Toscano I, Gomatzky C, de Goes PM, Junior AN, Claro JF, Pagani E (2009) Efficacy and tolerability of lodenafil carbonate for oral therapy in erectile dysfunction: a phase II clinical trial. *J Sex Med* 6:553–557
- Gratzke C, Ückert S, Kedia G, Reich O, Schlenker B, Seitz M, Becker AJ, Stief CG (2007) In vitro effects of PDE5 inhibitors sildenafil, vardenafil and tadalafil on isolated human ureteral smooth muscle: a basic research approach. *Urol Res* 35:49–54

- Greco EA, Spera G, Aversa A (2006) Combining testosterone and PDE5 inhibitors in erectile dysfunction: basic rationale and clinical evidences. *Eur Urol* 50:940–947
- Guay D, Hamel P, Blouin M, Brideau C, Chan CC, Chauret N, Ducharme Y, Huang Z, Girard M, Jones TR, Laliberte F, Masson P, McAuliffe M, Piechuta H, Silva J, Young RN, Girard Y (2002) Discovery of L-791,943: a potent, selective, non-emetic and orally active phosphodiesterase 4 inhibitor. *Bioorg Med Chem Lett* 12:1457–1461
- Guess HA (1995) Epidemiology and natural history of benign prostatic hyperplasia. *Urol Clin North Am* 22:247–261
- Gülcer C, Tüzel E, Dogantekin E, Kiziltepe G (2008) Does sildenafil affect uroflowmetry values in men with lower urinary tract symptoms suggestive of benign prostatic enlargement? *Urol Int* 80:181–185
- Gur S, Sikka SC, Hellstrom WJ (2008) Novel phosphodiesterase 5 (PDE5) inhibitors in the alleviation of erectile dysfunction due to diabetes and ageing-induced oxidative stress. *Expert Opin Investig Drugs* 17:855–864
- Gutierrez P, Hernandez P, Mas M (2005) Combining programmed intracavernous PGE1 injections and sildenafil on demand to salvage sildenafil non-responders. *Int J Impot Res* 17:354–358
- Han P, Zhu X, Michaeli T (1997) Alternative splicing of the high affinity cAMP-specific phosphodiesterase (PDE7A) mRNA in human skeletal muscle and heart. *J Biol Chem* 272:16152–16157
- Hatzimouratidis K, Hatzichristou DG (2008) Looking to the future for erectile dysfunction therapies. *Drugs* 68:231–250
- Heaton JP, Morales A, Owen J (1990) Topical glyceryltrinitrate causes measurable penile arterial dilatation in impotent men. *J Urol* 143:729–731
- Hieble JR, Ruffolo RR Jr (1996) The use of alpha-adrenoceptor antagonists in the pharmacological management of benign prostatic hyperplasia: an overview. *Pharmacol Res* 33:145–160
- Holmquist F, Stief CG, Jonas U, Andersson KE (1991) Effects of the nitric oxide synthase inhibitor NG-nitro-L-arginine on the erectile response to cavernous nerve stimulation in the rabbit. *Acta Physiol Scand* 143:299–304
- Jacobsen SJ, Guess HA, Panser L, Girman CJ, Chute CG, Oesterling JE, Lieber MM (1993) A population-based study of health care-seeking behaviour for treatment of urinary symptoms. *Arch Fam Med* 2:729–735
- Kalsi JS, Kell PD (2004) Update on the oral treatments for male erectile dysfunction. *J Eur Acad Dermatol Venereol* 18:267–274
- Kang KK, Yu JY, Yoo M, Kwon JW (2005) The effect of DA-8159, a novel PDE5 inhibitor, on erectile function in the rat model of hypercholesterolemic erectile dysfunction. *Int J Impot Res* 17:409–416
- Kaplan SA, Gonzalez RR, Te AE (2007) Combination of alfuzosin and sildenafil is superior to monotherapy in treating lower urinary tract symptoms and erectile dysfunction. *Eur Urol* 51:1717–1723
- Kühn R, Ückert S, Stief CG, Truss MC, Lietz B, Bischoff E, Schramm M, Jonas U (2000) Relaxation of human ureteral smooth muscle in vitro by modulation of cyclic nucleotide dependent pathways. *Urol Res* 28:110–115
- Küthe A, Mägert HJ, Ückert S, Forssmann WG, Stief CG, Jonas U (2000) Gene expression of the phosphodiesterases 3A and 5A in human corpus cavernosum penis. *Eur Urol* 38:108–114
- Küthe A, Wiedenroth A, Mägert HJ, Ückert S, Forssmann WG, Stief CG, Jonas U (2001) Expression of different phosphodiesterase genes in human cavernous smooth muscle. *J Urol* 165:280–283
- Liguori G, Trombetta C, de Giorgi G, Pomara G, Maio G, Vecchio D, Ocello G, Ollandini G, Bucci S, Belgrano E (2009) Efficacy and safety of combined oral therapy with tadalafil and alfuzosin: an integrated approach to the management of patients with lower urinary tract symptoms and erectile dysfunction. Preliminary report. *J Sex Med* 6:544–552
- Loughney K, Snyder PB, Uher L, Rosman GJ, Ferguson K, Florio VA (1999) Isolation and characterization of PDE10A, a novel human 3'.5'-cyclic nucleotide phosphodiesterase. *Gene* 234:109–117

- Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, Waldman SA (2000) Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 52:375–414
- Maurice DH, Palmer D, Tilley DG, Dunkerley HA, Netherton SJ, Raymond DR, Elbatany HS, Jimmo SL (2003) Cyclic nucleotide phosphodiesterase activity, expression, and targeting cells in the cardiovascular system. *Mol Pharmacol* 64:533–546
- Mazzu A, Nicholls A, Zinny M (2001) Vardenafil: a new highly selective PDE5 inhibitor, interacts minimally with nitroglycerin in healthy middle-aged subjects. *Int J Impot Res* 13(suppl 5):S64 (Abstract)
- McVary KT, Monnig W, Camps JL, Young JM, Tseng LJ, van den Ende G (2007a) Sildenafil citrate improves erectile function and urinary symptoms in men with erectile dysfunction and lower urinary tract symptoms associated with benign prostatic hyperplasia: a randomized, double-blind trial. *J Urol* 177:1071–1077
- McVary KT, Roehrborn CG, Kaminetsky JC, Auerbach SM, Wachs B, Young JM, Esler A, Sides GD, Denes BS (2007b) Tadalafil relieves lower urinary tract symptoms secondary to benign prostatic hyperplasia. *J Urol* 177:1401–1407
- Michaeli T, Bloom TJ, Martins T, Loughney K, Ferguson K, Riggs M, Rodgers L, Beavo JA, Wigler M (1994) Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase deficient *Saccharomyces cerevisiae*. *J Biol Chem* 268:12925–12932
- Oger S, Behr-Roussel D, Gorny D, Denys P, Lebret T, Alexandre L, Giuliano F (2007) Relaxation of phasic contractile activity of human detrusor strips by cyclic nucleotide phosphodiesterase type 4 inhibition. *Eur Urol* 51:772–780
- Ottensen B, Wagner G, Virag R, Fahrenkrug J (1984) Penile erection: possible role for vasoactive intestinal polypeptide as a neurotransmitter. *Br J Med* 288:9–11
- Porst H (1996) The rationale for prostaglandin E1 in erectile failure: a survey of worldwide experience. *J Urol* 155:802–815
- Rajasekaran M, Sikka S, Hellstrom W, Doherty P (1998) Evidence for the presence of the type 4 phosphodiesterase in isolated human cavernosal smooth muscle cells. *Int J Impot Res* 10(suppl 3): S55 (Abstract)
- Rajfer J, Aronson WJ, Bush PA, Dorey FJ, Ignarro LJ (1992) Nitric oxide as a mediator of relaxation of the corpus cavernosum in response to non-adrenergic, non-cholinergic neurotransmission. *N Engl J Med* 326:90–94
- Roehrborn CG, Marks LS, Fenter T, Freedman S, Tuttle J, Gittleman M, Morril B, Wolford ET (2004) Efficacy and safety of dutasteride in the four-year treatment of men with benign prostatic hyperplasia. *Urology* 63:709–715
- Rybulkin SD, Yan C, Bornfeld KE, Beavo JA (2003) Cyclic GMP phosphodiesterase and smooth muscle function. *Circ Res* 93:280–291
- Saighi D, Zerbib M, Thiounn N, Flam T, Conquy S, Jacob L, dall'Ava-Santucci J, Debré B, Dinh-Xuan AT (2000) In vitro study of the modulation of human ureteral tonus by nitric oxide and zaprinast, a phosphodiesterase inhibitor. *Prog Urol* 10:1161–1168
- Sairam K, Kulinskaya E, McNicholas TA, Boustead GB, Hanbury DC (2002) Sildenafil influences lower urinary tract symptoms. *BJU Int* 90:836–839
- Silver PJ, Dundore RL, Bode DC, de Garavilla L, Buchholz RA, van Aller G, Hamel LT, Bacon E, Singh B, Leshner GY (1997) Cyclic GMP potentiation by WIN 58237, a novel cyclic nucleotide phosphodiesterase inhibitor. *J Pharmacol Exp Ther* 271:1143–1149
- Smet PJ, Edyvane KA, Jonasvicius J, Marshall VR (1994) Colocalisation of nitric oxide synthase with vasoactive intestinal peptide, neuropeptide Y and tyrosine hydroxylase in nerves supplying the human ureter. *J Urol* 152:1292–1296
- Sommer F, Engelmann U (2004) Future options for combination therapy in the management of erectile dysfunction in older men. *Drugs Aging* 21:555–564
- Stief C, Porst H, Saenz de Tejada I, Ulbrich E, Beneke M, Vardenafil Study Group (2004) Sustained efficacy and tolerability with vardenafil over two years of treatment in men with erectile dysfunction. *Int J Clin Pract* 58:230–239

- Stief CG, Ückert S, Truss MC, Becker AJ, Machtens S, Jonas U (1996) A possible role for nitric oxide in the regulation of human ureteral smooth muscle tone in vitro. *Urol Res* 24:333–337
- Stief CG, Ückert S, Becker AJ, Truss MC, Jonas U (1998) Effects of specific phosphodiesterase (PDE) inhibitors on human and rabbit cavernous tissue in vitro and in vivo. *J Urol* 159:1390–1393
- Stief CG, Porst H, Neuser D, Beneke M, Ulbrich E (2008) A randomised, placebo-controlled study to assess the efficacy of twice-daily vardenafil in the treatment of lower urinary tract symptoms secondary to benign prostatic hyperplasia. *Eur Urol* 53:1236–1244
- Taher A, Schulz-Knappe P, Meyer M, Truss MC, Forssmann WG, Stief CG, Jonas U (1994) Characterization of cyclic nucleotide phosphodiesterase isoenzymes in the human ureter and their functional role in vitro. *World J Urol* 12:286–291
- Taher A, Meyer M, Stief CG, Jonas U, Forssmann WG (1997) Cyclic nucleotide phosphodiesterase isoenzymes in human cavernous smooth muscle. *World J Urol* 15:32–35
- Thadani U, Mazzu A (2002) Exercise-induced ischemia was not adversely affected by vardenafil during exercise stress test in men with coronary artery disease. *Eur Urol* 1(suppl 1):151 (Abstract)
- Toque HA, Teixeira CE, Lorenzetti R, Okuyama CE, Antunes E, de Nucci G (2008) Pharmacological characterization of a novel phosphodiesterase type 5 (PDE5) inhibitor, lodenafil carbonate, on human and rabbit corpus cavernosum. *Eur J Pharmacol* 591:189–195
- Truss MC, Becker AJ, Djamilian MH, Stief CG, Jonas U (1994) Role of the nitric oxide donor linsidomine chlorhydrate (SIN-1) in the diagnosis and treatment of erectile dysfunction. *Urology* 44:553–556
- Truss MC, Ückert S, Stief CG, Kuczyk MA, Jonas U (1996a) Cyclic nucleotide phosphodiesterase (PDE) isoenzymes in the human detrusor smooth muscle: I. Identification and characterization. *Urol Res* 24:123–128
- Truss MC, Ückert S, Stief CG, Kuczyk MA, Jonas U (1996b) Cyclic nucleotide phosphodiesterase (PDE) isoenzymes in the human detrusor smooth muscle: II. Effect of various PDE-inhibitors on smooth muscle tone and cyclic nucleotide levels in vitro. *Urol Res* 24:129–134
- Truss MC, Stief CG, Ückert S, Becker AJ, Wefer J, Schultheiss D, Jonas U (2001) Phosphodiesterase 1 inhibition in the treatment of lower urinary tract dysfunction: from bench to bedside. *World J Urol* 19:344–350
- Ückert S, Sandner P, Sigl K, Ulbrich E, Stief CG, Kuczyk MA (2009) Is there a role of the phosphodiesterase type 5 (PDE5) in the control of detrusor smooth muscle? A functional and molecular biology study. *J Urol* 181(suppl 4):152 (Abstract)
- Ückert S, Kütke A, Stief CG, Jonas U (2001a) Phosphodiesterase isoenzymes as pharmacological targets in the treatment of male erectile dysfunction. *World J Urol* 19:14–22
- Ückert S, Kütke A, Jonas U, Stief CG (2001b) Characterization and functional relevance of cyclic nucleotide phosphodiesterase isoenzymes of the human prostate. *J Urol* 166:2484–2490
- Ückert S, Hedlund P, Waldkirch E, Sohn M, Jonas U, Andersson KE, Stief CG (2004) Interactions between cGMP- and cAMP-pathways are involved in the regulation of penile smooth muscle tone. *World J Urol* 22:261–266
- Ückert S, Oelke M, Stief CG, Andersson KE, Jonas U, Hedlund P (2006) Immunohistochemical distribution of cAMP- and cGMP-phosphodiesterase (PDE) isoenzymes in the human prostate. *Eur Urol* 49:740–745

Evaluation of the Therapeutic Utility of Phosphodiesterase 5A Inhibition in the *mdx* Mouse Model of Duchenne Muscular Dystrophy

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Abstract Duchenne muscular dystrophy (DMD) is a devastating and ultimately fatal disease characterized by progressive muscle wasting and weakness. DMD is caused by the absence of a functional dystrophin protein, which in turn leads to reduced expression and mislocalization of dystrophin-associated proteins including neuronal nitric oxide (NO) synthase mu (nNOS μ). Disruption of nNOS μ signaling results in muscle fatigue and unopposed sympathetic vasoconstriction during exercise, thereby increasing contraction-induced damage in dystrophin-deficient muscles. The loss of normal nNOS μ signaling during exercise is central to the vascular dysfunction proposed over 40 years ago to be an important pathogenic mechanism in DMD. Recent preclinical studies focused on circumventing defective nNOS μ signaling in dystrophic skeletal and cardiac muscle by inhibiting phosphodiesterase 5A (PDE5A) have shown promising results. This review addresses nNOS signaling

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in normal and dystrophin-deficient muscles and the potential of PDE5A inhibition as a therapeutic approach for the treatment of cardiovascular deficits in DMD.

Keywords Cardiac muscle · Cardiomyopathy · cGMP · Duchenne Muscular Dystrophy · Dystrophin · Mdx · Nitric oxide · nNOS · Neuronal nitric oxide synthase · PDE5 · PDE5 inhibitors · Sildenafil · Skeletal muscle

1 Introduction

The absence of normal neuronal nitric oxide (NO) synthase mu (nNOS μ) signaling in the muscles of humans and mice is a well-described consequence of the loss of dystrophin, the primary cause of Duchenne Muscular Dystrophy (DMD). The absence of nNOS μ signaling impairs blood supply to contracting skeletal muscles, exposing working muscles to continuous damaging ischemic insult (Thomas et al. 1998; Asai et al. 2007). At present, it is not possible to selectively increase nNOS expression or activity using a pharmacological approach, but it is possible to mimic some of the effects of increased nNOS activity, which increases synthesis of cGMP and cGMP signaling (or circumvent aberrant upstream nNOS signaling) by inhibiting the activity of downstream cGMP-hydrolyzing phosphodiesterases (PDEs).

Recent efforts to pharmacologically enhance nNOS-cGMP signaling in dystrophic muscles have involved the targeted inhibition of PDE5A with sildenafil (Viagra[®], Revatio[®]) or tadalafil (Cialis[®]), commonly used to treat erectile dysfunction and pulmonary hypertension. In the presence of a stimulus of cGMP synthesis such as NO, PDE5A inhibitors block cGMP breakdown, thereby raising cellular cGMP concentrations in many tissues such as smooth muscle. Inhibition of PDE5A is a highly attractive therapeutic approach for treating DMD for at least five reasons. First, there is strong evidence for pronounced vascular dysfunction in DMD, particularly reduced blood delivery to active muscle (Mendell et al. 1971; Thomas et al. 1998; Sander et al. 2000). Second, in several animal models, PDE5A inhibition has been shown to provide beneficial effects on skeletal, smooth, and cardiac muscle tissues (Asai et al. 2007; Khairallah et al. 2008; Reffelmann and Kloner 2009). Third, the ability to treat all muscle tissues is very important since therapeutic approaches that correct only skeletal muscle dysfunction increase cardiac workload, which is damaging to the weakened dystrophic heart (Townsend et al. 2008). Those approaches that fail to address vascular dysfunction resulting from impaired nNOS signaling in smooth muscle result in significant unopposed sympathetic vasoconstriction known to exacerbate skeletal muscle damage (Ito et al. 2006). Fourth, increases in cGMP levels or PDE5A inhibition confer substantial cardioprotective effects in several animal models including improved diastolic dysfunction (Takimoto et al. 2005; Das et al. 2008; Reffelmann and Kloner 2009). This is an important consideration since existing treatments for DMD-associated cardiomyopathy, including angiotensin-converting enzyme (ACE) inhibitors and β -adrenergic blockade, predominantly address systolic, but not the diastolic dysfunction in DMD patients

(Markham et al. 2006). Fifth, an obvious practical advantage is the availability of FDA-approved, potent and highly selective PDE5A inhibitors that include Viagra[®]/Revatio[®] (sildenafil), Levitra[®] (vardenafil), and Cialis[®] (tadalafil). These drugs have already been extensively tested in normal adults, while sildenafil has also been tested in children. Thus, testing of safety and efficacy in DMD and other muscular dystrophies in humans could occur quite quickly, which is an important consideration for a rapidly progressing and invariably fatal disease. Before discussing studies of the impact of PDE5A inhibition on the dystrophic pathology of the *mdx* mouse model of DMD, we first briefly outline what is known about the function of nNOS-derived NO-signaling pathways in normal and dystrophin-deficient muscles.

2 nNOS Signaling in Skeletal Muscle

The free radical gas NO is indispensable for normal muscle health and exercise performance. In skeletal muscle, neuronal nitric oxide synthase (nNOS) isozymes are the predominant sources of NO. nNOS isozymes are Ca²⁺/calmodulin-regulated, heme-containing flavoproteins that synthesize gaseous NO from L-arginine, in an NADPH and O₂-dependent manner (Bredt and Snyder 1990; Stuehr et al. 2004). Skeletal muscles express at least two alternatively spliced forms of nNOS called nNOS μ and nNOS β (Silvagno et al. 1996; Percival et al. 2010; Fig. 1). nNOS μ is localized to the sarcolemmal and to an undefined cytosolic compartment, whereas nNOS β is localized to the Golgi complex (Brenman et al. 1995, 1996; Thomas et al. 2003; Percival et al. 2010; Fig. 1). nNOS μ contains an amino terminal PDZ (PSD-95, discs-large, ZO-1) domain and an internal short sequence (mu insert) of unknown function, while nNOS β lacks both the PDZ domain and the mu insert. Instead, nNOS β contains a short unique amino terminal sequence that is a putative Golgi-targeting motif. Thus, exon choice at the amino terminus appears to regulate the differential targeting of these two nNOS isozymes. Sarcolemmal localization of nNOS μ requires the correct expression and localization of dystrophin, α -syntrophin, and α -dystrobrevin (Brenman et al. 1995, 1996; Adams et al. 2000), all of which are members of the dystrophin glycoprotein complex (DGC). Thus, skeletal muscle has at least two NO-signaling compartments defined by the localization of the two NOS isoenzymes to either the subsarcolemmal space or Golgi membranes.

While nNOS μ and nNOS β are differentially localized, they both synthesize NO, which exerts its regulatory effects through cGMP-dependent and cGMP-independent pathways. cGMP (guanosine 3':5'-cyclic monophosphate) is an important second messenger produced by the NO receptor, soluble guanylyl cyclase (sGC) (Mergia et al. 2009). NO binds at multiple sites including the critical heme group within sGC, stimulating it to convert guanosine triphosphate (GTP) into cGMP. In turn, cGMP binds and activates downstream effectors including: cGMP-dependent protein kinases (PKG, also known as cGK), cyclic nucleotide-gated (CNG) channels, and cGMP-regulated PDEs (Hofmann et al. 2009; Craven and Zagotta 2006; Bender and Beavo 2006). In skeletal muscle, sGC and PKG isoforms are localized

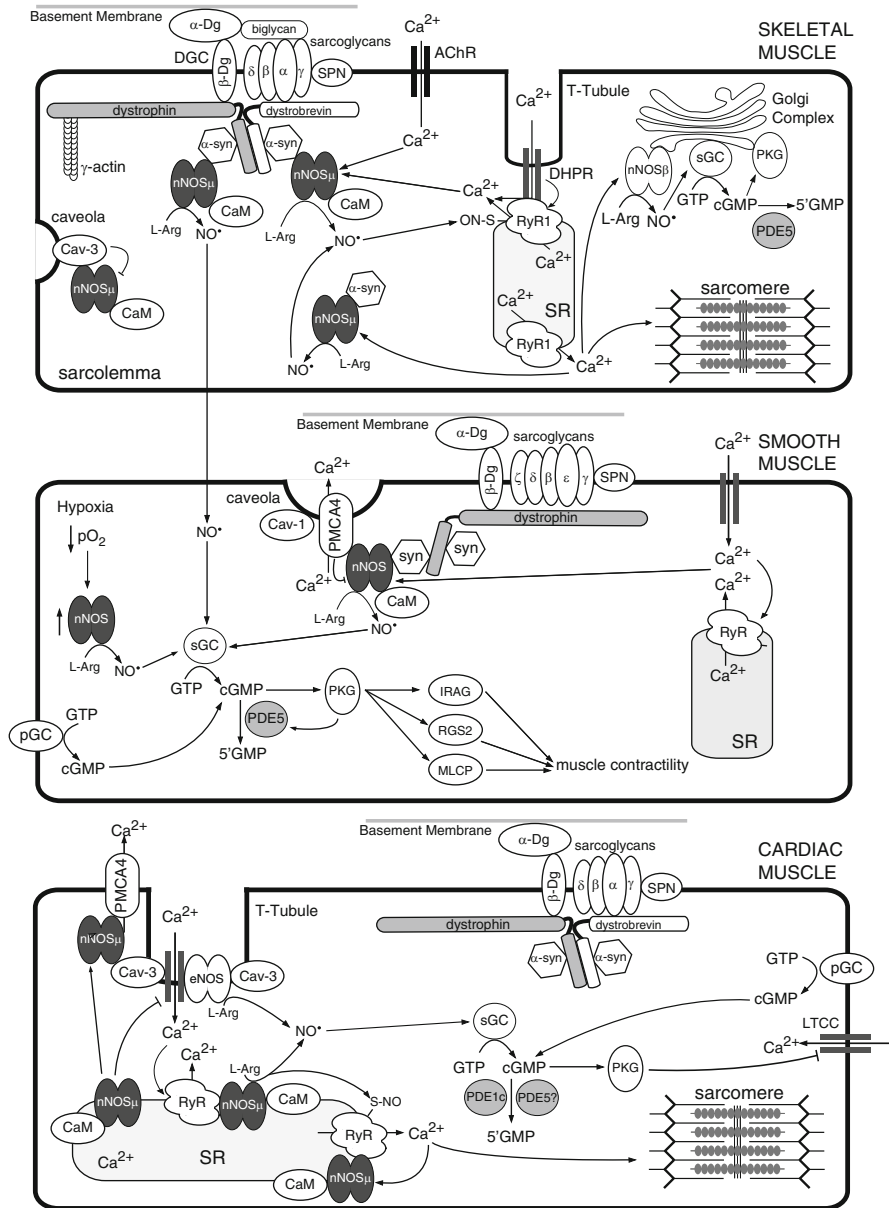


Fig. 1 Propagation of NO-cGMP signals in skeletal, smooth, and cardiac muscle. Nitric oxide synthase enzymes (nNOS and eNOS) regulate, and are regulated by, Ca^{2+} fluxes in muscle cells. Ca^{2+} /CaM activation of nNOS (or eNOS) leads to synthesis of NO, which in turn binds and activates sGC. cGMP produced by sGC then modulates downstream effector activity (see text) Abbreviations: α -Dg, α -dystroglycan; β -Dg, β -dystroglycan; α -syn, α -syntrophin; AChR, nicotinic acetylcholine receptor; CaM, calmodulin; Cav-1, Caveolin-1; Cav-3, caveolin-3; DHPR, dihydropyridine receptor; IRAG, inositol 1,4,5-triphosphate receptor I-associated cGMP

to the Golgi complex (Percival et al. 2010; Fig. 1). PKG is also localized to the neuromuscular junction (Chao et al. 1997). NO can also act through cGMP-independent pathways by directly reacting with thiol residues of cysteine groups. This NO-based posttranslational modification, known as S-nitrosylation, is also an important signal transduction mechanism. For example, the activity of skeletal muscle RyR1, the ryanodine Ca^{2+} release channel, is positively regulated by nitrosylation (Eu et al. 2000, Fig. 1). Therefore, in skeletal muscle, nNOS-synthesized NO signals can be propagated through both cGMP-dependent and cGMP-independent mechanisms.

Rapid modulation of NO-cGMP signaling is mediated both by the rate of cGMP synthesis and by cGMP degradation by cGMP-PDEs. Several different PDEs are able to hydrolyze cGMP including PDEs 1, 2, 3, 5, 6, 9, and 11. cGMP-specific PDEs, such as PDE5A, hydrolyze only cGMP, thereby decreasing the cellular levels of cGMP (Bender and Beavo 2006; Fig. 1). Inhibition of these cGMP-hydrolyzing PDEs can raise cGMP levels and effectively amplify the upstream NO signal. One of the most studied cGMP-PDEs is PDE5A, which is predominantly expressed in the vascular smooth muscle cells (VSMCs) of most vascular beds, fibroblasts, and myofibroblasts (Wallis et al. 1999). PDE5A-mediated cGMP degradation promotes smooth muscle contraction with concomitant blood vessel constriction. Active PDE5A is also expressed in skeletal muscle homogenates and cell lines including C2C12 myoblasts and myotubes (Bloom 2002; unpublished observations). Although PDE5A expression in cardiomyocytes has been reported, this issue is contentious since others contend there is no significant PDE5A expression or activity in these cells (Senzaki et al. 2001; Takimoto et al. 2005; Reffelmann and Kloner 2009; Lukowski et al. 2010). Thus, PDE5A is expressed in skeletal and smooth muscles and perhaps at very low levels in cardiomyocytes. nNOS is also expressed in VSMCs and cardiomyocytes (Xu et al. 1999; Ward et al. 2005). In VSMCs, nNOS promotes smooth muscle relaxation and blood vessel dilation, particularly during chronic hypoxia (Ward et al. 2005). It is clear that skeletal, cardiac, and smooth muscle cells possess the necessary molecular machinery for localized nNOS-cGMP signaling (Fig. 1). Importantly, inhibition of PDE5 activity provides a general approach to amplify nNOS-mediated signal transduction, or to broadly enhance NO-cGMP signaling activity, particularly in smooth muscle cells.

Recent studies of NO signaling in skeletal muscle have provided new insights into nNOS function. nNOS μ participates in pathways that regulate (1) contraction-induced glucose uptake and glucose homeostasis, (2) muscle mass and atrophy (3) mitochondrial integrity (4) susceptibility to fatigue (5) postexercise strength (6) exaggerated exercise-induced inactivity, (7) and blood delivery during exercise

Fig. 1 (continued) kinase substrate; PKG, protein kinase G (cGK); L-arg, L-arginine; LTCC, L-type calcium channel; MLCP, myosin light chain phosphatase; NO, nitric oxide; PDE, phosphodiesterase; pGD, particulate guanylyl cyclase; PMCA4, plasma membrane calcium ATPase 4; RGS2, regulator of G protein signaling 2; RyR, ryanodine receptor; sGC, soluble guanylyl cyclase; SPN, sarcospan; SR, sarcoplasmic reticulum

(Thomas et al. 1998; Firestein and Bredt 1999; McConell and Wadley 2008; Suzuki et al. 2007; Percival et al. 2008, 2010; Kobayashi et al. 2008; Wehling-Henricks et al. 2009). Therefore, nNOS μ appears to control physiological pathways that collectively regulate metabolic energy flux, particularly during muscle contraction. These roles also support the proposition that muscle nNOS μ function is most important under conditions of physiological stress, particularly prolonged inactivity or exercise. In agreement with this proposition, the muscles of trained athletes express higher levels of nNOS μ , while nNOS μ levels are lower in less active or sedentary muscles and often absent in myopathic muscles; therefore, establishing a close relationship between nNOS μ expression and muscle activity (Brenman et al. 1995; Chang et al. 1996; Chao et al. 1996; Crosbie et al. 2002; McConell et al. 2007; Suzuki et al. 2007; Kobayashi et al. 2008).

The exercise performance of muscle is highly dependent on oxygen supply. Perhaps, the best studied function of nNOS μ is its ability to attenuate resistance vessel vasoconstriction, matching oxygen delivery with demand during muscle contraction (Thomas and Victor 1998; Thomas et al. 1998, 2003). The localization of nNOS μ to the sarcolemma is critical for this vasomodulatory function and cannot be compensated for by cytoplasmic nNOS μ or Golgi nNOS β (Thomas et al. 2003; Percival et al. 2010). Taken together, these data demonstrate a role for nNOS μ in regulating oxygen delivery during muscle contraction and strongly support a role for nNOS μ in regulating the exercise performance of skeletal muscle.

3 nNOS Signaling in Cardiac Muscle

As in skeletal muscle, nNOS μ -synthesized NO in the heart has emerged as an important autocrine regulator of cardiomyocyte contractility and coronary blood flow (Barouch et al. 2002; Sears et al. 2003; Zhang et al. 2008; Seddon et al. 2009, Fig. 1). Cardiac nNOS μ plays an essential role in promoting relaxation of the myocardium and may do so via the regulation of Ca²⁺ flux. For example, nNOS μ -derived NO decreases inward Ca²⁺ movement (thereby reducing basal contractility) by negatively regulating the activity of the L-type Ca²⁺ channel (Sears et al. 2003). However, in contrast to its distribution in skeletal muscle, nNOS μ is primarily localized to the sarcoplasmic reticulum in cardiac myocytes in a complex with the ryanodine receptor Ca²⁺-release channel (RyR2), suggesting tissue-specific differences in nNOS μ function in excitation-contraction coupling (Xu et al. 1999; Sears et al. 2003; Fig. 1). Cardiac nNOS μ is thought to serve a cardioprotective role under conditions of pathophysiological stress. For example, nNOS μ translocation to the sarcolemma occurs during myocardial infarction and heart failure, where it blunts β -adrenergic signaling and reduces cardiac contractility (Bendall et al. 2004). Additional support for a cardioprotective role comes from findings that nNOS μ depletion exacerbates maladaptive cardiac remodeling following myocardial infarction (Saraiva et al. 2005). These data strongly support an

important role for nNOS μ in the regulation of cardiomyocyte contractility and Ca²⁺ flux, functions that are protective in a pathophysiologically distressed heart.

4 Skeletal Muscle Pathogenesis in Duchenne Muscular Dystrophy

Skeletal muscle nNOS μ expression, localization, and signaling are severely disrupted in DMD, an X-linked muscle wasting disease that occurs in 1 in every 3,600–6,000 live male births (Davies and Nowak 2006; Bushby et al. 2009). DMD patients exhibit elevated serum creatine kinase activity levels (due to increased sarcolemmal permeability) and progressive muscle wasting and weakness leading to loss of ambulation by 12 years of age (Davies and Nowak 2006). Voluntary limb and trunk muscles are initially affected, followed by respiratory and cardiac muscle involvement. DMD results predominantly from frame shift mutations in the gene encoding dystrophin (Hoffman et al. 1987).

Dystrophin is a 427 kDa rod-shaped actin-binding protein that resides at the cytoplasmic face of the sarcolemma (Hoffman et al. 1987; Koenig et al. 1988). It is the namesake of the DGC (dystrophin glycoprotein complex), a multiprotein complex that links the extracellular basal lamina with the intracellular γ -actin microfilament system (Ervasti and Campbell 1993). Dystrophin stabilizes myofibers against mechanical forces generated during muscle contraction (Ervasti 2007). The DGC appears to have a mechanotransduction role whereby dystrophin is necessary for inhibition of stretch-activated Ca²⁺ channel activity (Vandebrouck et al. 2001). As mentioned above, dystrophin also serves as a scaffold on which signaling molecules are localized primarily by the adaptor protein α -syntrophin (Percival et al. 2006). nNOS μ is the best characterized ligand of α -syntrophin (Brenman et al. 1996; Kameya et al. 1999; Adams et al. 2000). The mode of nNOS μ binding to α -syntrophin leaves the PDZ domain of nNOS μ free to bind other proteins including phosphofruktokinase (Brenman et al. 1996; Hillier et al. 1999; Firestein and Bredt 1999; Adams et al. 2001). Dystrophin deficiency leads to the loss of α -syntrophin and nNOS μ from the DGC. Mislocalized nNOS μ fails to override exercise-induced sympathetic vasoconstriction (Thomas et al. 2003). Thus, the loss of dystrophin simultaneously impacts muscle structural integrity and uncouples contraction-induced signaling, including NO-mediated signal transduction.

Disruption of nNOS μ expression and signaling occurs not only in DMD, but also in other myopathies, including: Becker Muscular Dystrophy (also from less pathogenic mutations of dystrophin), Limb Girdle Muscular Dystrophies 2C, 2D, and 2E (resulting from mutations of γ -, α -, and β -sarcoglycan, respectively), and Ulrich Congenital Muscular Dystrophy (collagen VI mutation) (Brenman et al. 1995; Chang et al. 1996; Chao et al. 1996; Crosbie et al. 2002; Kobayashi et al. 2008). These myopathies are all characterized by the absence of sarcolemmal nNOS μ protein expression, whereas both cytosolic and sarcolemmal nNOS μ expression are

substantially reduced ($\geq 80\%$) in dystrophin-deficient muscles (Chang et al. 1996). Thus, dystrophin is necessary for the normal expression and localization of nNOS μ . The mechanisms by which nNOS μ signaling is disrupted remain to be determined in other myopathies. In summary, it is clear that nNOS signaling abnormalities are common to a broad spectrum of muscle diseases.

In addition to the dysregulation of nNOS μ , many other proteins and pathways are deregulated in dystrophin-deficient muscle. The loss of dystrophin increases muscle instability and permeability, reflected by excessive Ca^{2+} influx. In turn, Ca^{2+} overload leads to activation of proteases and mitochondrial dysfunction causing muscle necrosis and cycles of muscle cell degeneration and regeneration (Davies and Nowak 2006). Regeneration is easily observed histologically as clusters of centrally nucleated fibers. Muscle breakdown is accompanied by infiltration of inflammatory cells, particularly macrophages. Initially, the regenerative capacity of dystrophin-deficient muscle keeps pace with degeneration, but is soon exhausted and myofibers are gradually replaced by adipose and fibrous connective tissue (Davies and Nowak 2006).

Corticosteroid treatment, despite significant side effects and limited efficacy, is the mainstay therapy for the preservation of skeletal muscle function in DMD (Manzur et al. 2008). Death typically ensues in the third decade of life with 75% of DMD patients dying from respiratory failure while the remainder succumbs to heart failure (Finsterer and Stöllberger 2003). However, the incidence and severity of cardiac dysfunction are on the rise because of improvements in noninvasive ventilatory support (Eagle et al. 2007). Better understanding of the cascade of pathological changes in dystrophin-deficient muscles is necessary to identify new targets for therapeutic pharmacological intervention in the short term.

The effects of dystrophin-deficiency have been extensively studied in the *mdx* mouse, an animal model for DMD. As is the case in humans, *mdx* mice exhibit significant skeletal muscle weakness, susceptibility to fatigue, exercise intolerance, shorter lifespan, elevated serum creatine kinase activity, widespread muscle degeneration and regeneration, muscle necrosis, Ca^{2+} overload, fibrosis, and inflammation (Stedman et al. 1991; Davies and Nowak 2006; Chamberlain et al. 2007; Willmann et al. 2009). Dystrophin-deficient *mdx* myofibers exhibit a characteristic susceptibility to lengthening contraction that may result from inappropriate stretch-activated Ca^{2+} channel activity (Petrof et al. 1993; Dellorusso et al. 2001; Whitehead et al. 2006). The diaphragm muscle in *mdx* mice best recapitulates the pathology observed in the skeletal muscles from DMD patients (Stedman et al. 1991). However, mice are less affected by the absence of dystrophin than humans and exhibit a slower and milder disease progression. Highly effective compensatory mechanisms are clearly at play in dystrophin-deficient tissues of *mdx* mice. Increased expression of utrophin, a functional paralogue of dystrophin, is thought to play a significant compensatory role (Davies and Nowak 2006). Overall, however, *mdx* mice represent a useful model for investigating dystrophic pathology and for evaluating the efficacy of experimental treatments.

5 Cardiac Muscle Pathogenesis in Duchenne Muscular Dystrophy

Like skeletal muscle, cardiac muscle is also severely affected by the loss of dystrophin with cardiomyopathy beginning early in DMD patients. Left ventricle (LV) dysfunction is readily detectable in patients in their teens. By 18 years of age, clinically relevant symptoms of cardiomyopathy are evident in 90% of patients (Chenard et al. 1993; de Kermaecq et al. 1994; Finsterer and Stöllberger 2003). Substantial improvements in noninvasive respiratory support have extended patient longevity, but are accompanied by increased incidence of severe cardiac dysfunction (Eagle et al. 2007). This has necessarily led to an upturn in the management of cardiac dysfunction that now predominates in the late stages of DMD.

In contrast to skeletal muscle, the impact of dystrophin-deficiency on cardiac function in humans has received much less attention. Over time, a DMD-associated dilated cardiomyopathy (DCM) can develop as a consequence of the absence of dystrophin from the heart (Finsterer and Stöllberger 2003). Similar to skeletal muscle, the absence of dystrophin in the cardiomyocyte enhances membrane permeability leading to Ca^{2+} overload. Pathologically, high intracellular Ca^{2+} concentrations result in excessive protease activity and impair mitochondrial oxidative phosphorylation, causing widespread cardiomyocyte necrosis. Cardiomyocyte death results in inflammation and extensive fibrosis, particularly of the LV wall, reducing ventricular compliance and diastolic function (Moriuchi et al. 1993; Frankel and Rosser 1976). Atrial and ventricular chamber walls stretch and thin out due to cardiomyocyte death, leading to chamber dilation. As a result, both systolic and diastolic function of the dystrophin-deficient heart is impaired. Eventually, DCM develops into congestive heart failure. DCM may be associated with arrhythmias and other electrical impulse conduction defects that also contribute to cardiac dysfunction (Finsterer and Stöllberger 2003; Thrush et al. 2009). Sinus tachycardia (abnormally fast heart rate) is a prevalent arrhythmia in DMD. Short PR intervals, tall R waves, and prominent Q waves are also common impulse conduction abnormalities (Finsterer and Stöllberger 2003; Thrush et al. 2009).

In the clinic, ACE inhibitors and β -adrenergic blockade provide the mainstay of therapeutic intervention in DMD (Finsterer and Stöllberger 2003). While these interventions are often effective for enhancing systolic function in DMD hearts, diastolic dysfunction is left largely unaddressed. This is an important point relevant to the potential utility of PDE5A inhibitors in treating DMD-associated DCM since sildenafil, currently used to treat erectile dysfunction and pulmonary hypertension, will be tested in large clinical study for efficacy in treating diastolic dysfunction in heart failure (ClinicalTrials.gov Identifier: NCT00781508). Despite current interventions, DMD deaths from cardiac failure are on the rise and according to some estimates, death from congestive heart failure and/or arrhythmias can account for 20–50% of deaths in DMD (Finsterer and Stöllberger 2003; Ishikawa et al. 1999).

Thus, dystrophin-deficiency has serious negative consequences for cardiac as well as skeletal muscle function.

Unlike skeletal muscle, the role of nNOS in the dystrophic heart has not been investigated in humans. Also, studies of NO-cGMP signaling in *mdx* mouse hearts are limited. Knowledge of the NOS-signaling pathway activity in dystrophy is very important from a therapeutic standpoint, since NO is required for many of the cardioprotective effects of sildenafil (Nagayama et al. 2008). In the absence of NO, sGC may not be sufficiently active and, consequently, fails to produce physiologically sufficient amounts of cGMP, rendering the inhibition of cGMP-hydrolyzing PDEs therapeutically ineffective (Fernhoff et al. 2009).

nNOS activity, but not nNOS protein expression, is inhibited in dystrophin-deficient cardiac muscle. Cardiac nNOS μ levels appear unaffected by dystrophin-deficiency, despite a significant reduction in nNOS μ activity (Bia et al. 1999; Wehling-Henricks et al. 2005). The activity of endothelial NO synthase (eNOS), the other NO-generating enzyme in cardiomyocytes, is unaffected by the absence of dystrophin (Bia et al. 1999). Elevated expression of atrial natriuretic peptide (the ligand for the transmembrane guanylyl cyclase ANP receptor A [NPR-A]) mRNA in *mdx* hearts is consistent with deficits in cGMP signaling in dystrophic cardiac tissue (Khairallah et al. 2007) and suggests that increased ANP pathway activity may be a compensatory mechanism in the *mdx* heart.

Although the impact of dystrophin-deficiency on cardiac function has received less attention than in skeletal muscles, it is clear that *mdx* mice develop a less severe, but pronounced cardiomyopathy compared with humans. Two important distinctions between *mdx* and DMD hearts are that ventricular fibrosis is less extensive and chamber dilation is not pronounced in murine *mdx* hearts (Quinlan et al. 2004; Wehling-Henricks et al. 2005; Spurney et al. 2008). The reasons for these differences are unknown. However, as in humans, the absence of dystrophin initiates a similar cascade of pathological events that leads to increased membrane permeability and Ca²⁺ overload, culminating in cardiomyocyte necrosis and death. Increased cardiomyocyte death likely results in part from an increased susceptibility to contraction-induced damage (Danialou et al. 2001). The predisposition of dystrophin-deficient cardiomyocytes to necrosis and mechanical stress results in significant contractile dysfunction.

Early *mdx* mouse studies suggested that cardiomyopathy could only be detected around 8 months of age (Quinlan et al. 2004). However, recent studies have demonstrated cardiac dysfunction including LV systolic and diastolic dysfunction in mice as young as 8–10 weeks of age (Danialou et al. 2001; Wu et al. 2008; Khairallah et al. 2007). Cardiac dysfunction is not evident from noninvasive in vivo analysis at these young ages, suggesting compensatory mechanisms in vivo. These findings are consistent with the progression of DCM in DMD patients, where early cardiomyopathy goes largely unnoticed, only becoming clinically symptomatic in the second decade of life. Hearts from 9- to 10-month-old *mdx* mice exhibit marked systolic dysfunction and pathological LV remodeling (Quinlan et al. 2004; Spurney et al. 2008). Also, myocardial performance index (MPI) is significantly increased, indicative of increased cardiac

dysfunction (Spurney et al. 2008). Thus, collectively, these data indicate pronounced left ventricular dysfunction in *mdx* hearts.

As observed in humans, contractile dysfunction is often accompanied by arrhythmias and abnormal electrical impulse conduction. Electrocardiographic studies reveal that *mdx* and DMD hearts exhibit similar aberrant impulse interval characteristics including deep Q waves, a decreased S:R wave ratio, polyphasic R waves, shortened PR interval and QTc intervals and cardiac arrhythmias, such as premature ventricular contractions (Chu et al. 2002; Wehling-Henricks et al. 2005; Bostick et al. 2009). In summary, dystrophin-deficiency negatively affects cardiomyocyte survival and function in *mdx* mice. Furthermore, *mdx* hearts recapitulate key features of cardiomyopathy in DMD hearts including cardiomyocyte necrosis, susceptibility to mechanical stress, diastolic dysfunction, systolic dysfunction, and impulse propagation defects.

6 Vascular Dysfunction Contributes to the Pathogenesis of DMD

While research in DMD has primarily focused on dystrophin function in skeletal muscle and, to a lesser extent, cardiac muscle, smooth muscle dysfunction may also play a role in the dystrophic phenotype. Both dystrophin and nNOS μ are expressed in VSMCs (Ward et al. 2005; Ito et al. 2006; Fig. 1). Indeed, abnormalities in smooth muscle in *mdx* mice appear to contribute to the dystrophic phenotype, for example, by impairing blood supply during exercise (Ito et al. 2006). This is particularly relevant to the therapeutic utility of PDE5 inhibitors in dystrophy because PDE5A is highly expressed in VSMCs in the vascular beds of the circulatory system; thus, any consideration of the effects of systemic PDE5A inhibition on *mdx* pathology must also consider any potential impact on VSMC function (Wallis et al. 1999). Furthermore, vascular dysfunction has long been suspected to contribute to the dystrophic pathology of DMD (Mendell et al. 1971).

Evidence of vascular dysfunction, specifically small clusters of necrotic fibers, was first observed in skeletal muscle biopsies from DMD patients over 40 years ago. This muscle pathology could be recapitulated by vascular obstruction, suggesting that muscle necrosis could result from defects in a shared blood vessel. This reasoning formed the basis for the vascular hypothesis proposed by Engel and coworkers that stated that skeletal muscle microcirculation dysfunction could account for the pathogenesis of DMD (Mendell et al. 1971). This hypothesis was largely abandoned when no structural abnormalities of the vasculature were found (Jerusalem et al. 1974). However, the vascular theory was revisited when Victor and coworkers demonstrated that α -adrenergic receptor-mediated sympathetic vasoconstriction was unopposed in the exercising hind limbs of *mdx* and KN1 (nNOS knockout 1) mice and contracting forearms of DMD patients (Thomas et al. 1998; Sander et al. 2000). Thus, during exercise, dystrophin-deficient muscles

lacking sarcolemmal nNOS μ would be subjected to repeated rounds of ischemia (functional ischemia) causing myofiber damage in dystrophin-deficient muscles and contributing to the profound exercise intolerance observed in DMD patients. The loss of nNOS μ targeting provides a mechanism for the vascular dysfunction observed in dystrophin-deficient muscle.

Direct evidence that reduced blood flow in postcontraction muscles played a primary role in the disease pathogenesis came from *in vivo* microscopy studies. Pretreatment of dystrophic *mdx* sternomastoid muscle with the NO donor SNAP (S-nitroso-*N*-acetylpenicillamine), or the cGMP analog 8-CPT-cGMP (8-chlorophenylthio-cGMP) reversed the ischemic effects of primary arteriole constriction and prevented contraction-induced myofiber damage (Asai et al. 2007). This study provided proof of principle that pharmacological augmentation of NO-cGMP signaling to increase blood supply to active muscles can reduce postexercise muscle damage. The key role of sarcolemmal nNOS μ in preventing ischemic damage in dystrophic muscle was later confirmed by a study of the therapeutic capability of a Δ H2-R15 minidystrophin cDNA that could restore sarcolemmal nNOS μ expression in *mdx* mice (Lai et al. 2009). Sarcolemmal nNOS μ expression restored the ability of *mdx* muscle to oppose exercise-induced sympathetic vasoconstriction, prevented contraction-induced fiber degeneration and macrophage infiltration and improved exercise performance (Lai et al. 2009). Thus, contraction-induced sarcolemmal nNOS μ -derived NO signaling to the VSMC of adjacent resistance vessels plays an important role in preserving skeletal muscle integrity.

While the importance of contraction-triggered paracrine signaling from skeletal muscle nNOS μ -synthesized NO to the adjacent vasculature is apparent, dystrophin-associated nNOS in VSMC of blood vessels may also facilitate opposition to sympathetic vasoconstriction during exercise (Fig. 1). In *mdx* mice, the loss of dystrophin leads to the reduction of nNOS μ isoenzyme expression in both skeletal and vascular smooth muscle (Ito et al. 2006). It is not clear whether the nNOS isozyne in question is nNOS μ or nNOS α since both may be expressed in VSMCs (Boulanger et al. 1998; Ward et al. 2005). Nevertheless, increased smooth muscle specific-expression of dystrophin in *mdx* mice restored nNOS protein expression and provided an intermediate level of inhibition of vasoconstriction during contraction (Ito et al. 2006). Smooth muscle-specific dystrophin expression in *mdx* mice reduced serum CK levels, indicating decreased myofiber permeability (Ito et al. 2006). Unlike the paracrine action of sarcolemmal nNOS μ -derived NO on adjacent blood vessels, VSMC nNOS-derived NO acts in an autocrine fashion to promote smooth muscle cGMP-dependent relaxation and vasodilation. Together, these data suggest that aberrant nNOS signaling in VSMCs can also contribute to the microvascular dysfunction and dystrophic pathology by increasing myofiber permeability and susceptibility to ischemic damage during exercise. Thus, the vascular dysfunction in *mdx* mice likely results from simultaneous disruption of nNOS isozyne expression and signaling in both skeletal and smooth muscle.

7 Augmentation of Nitric Oxide Signaling in *mdx* Mice

The observations that nNOS μ signaling is disrupted in dystrophin-deficient skeletal, cardiac, and smooth muscle cells, combined with abnormal nNOS and cGMP signaling in dystrophin-deficient cardiac muscles, provides a compelling rationale for the use of approaches that enhance nNOS signaling to treat dystrophinopathies. To date, such approaches have used exogenous sources of NO to enhance NO signaling, such as NO synthase transgenes or NO donors. Other approaches have focused on modulating downstream effector activity, specifically by inhibiting PDE5A activity to enhance cGMP levels that normally result from NO-mediated activation of sGC.

Targeting nNOS signaling pathways has proved efficacious for reducing muscle damage and improving exercise performance in the *mdx* mouse model of DMD. Reengineering of conventional minidystrophin gene therapy cassettes to restore sarcolemmal nNOS μ expression provides significant additional improvements over conventional microdystrophin cassettes, including enhanced vasomodulation, exercise performance, and resistance to exercise-induced muscle damage (Lai et al. 2009). Thus, the ability to restore sarcolemmal nNOS μ expression substantially improves gene therapy-based intervention in *mdx* mice. These data are consistent with reduced contraction-induced myofiber damage observed after the application of NO donors (Asai et al. 2007). Cytosolic expression of an nNOS α transgene reduced muscle damage without affecting membrane permeability in adult *mdx* mice (Wehling et al. 2001). Substantial reductions in macrophages densities and cytolytic activity were observed, suggesting that the anti-inflammatory properties of NO were responsible for the reduction in muscle damage (Wehling et al. 2001). In agreement with this proposal, a compensatory increase in utrophin expression was not observed (Tidball and Wehling-Henricks 2004). Transduction of *mdx* skeletal muscles by adenovirus carrying a constitutively active eNOS gene also improved dystrophic pathology and increased myofiber size by upregulating follistatin expression (Colussi et al. 2008). Histone deacetylase 2 (HDAC 2) inhibition by NO-dependent S-nitrosylation is responsible for the anti-dystrophic impact of increased cytosolic NO, highlighting a novel link between NO signaling and chromatin remodeling in dystrophic skeletal muscle (Colussi et al. 2008). These findings provide evidence that NO can also exert beneficial effects on dystrophic muscle through cGMP-independent pathways. Taken together, these studies provide a compelling rationale for the potential therapeutic utility of increasing NO-cGMP signaling in dystrophin-deficient tissues.

As in skeletal muscle, cardioprotective effects of increased NO concentrations have been reported in *mdx* cardiac muscle. Cardiomyocyte-specific expression of a nNOS α transgene in *mdx* mice significantly reduced interstitial fibrosis (Wehling-Henricks et al. 2005). Interestingly, ectopic expression of nNOS α in *mdx* hearts (nNOS α is not normally expressed in cardiomyocytes) corrected common impulse conduction defects including deep Q waves, a decreased S:R wave ratio, polyphasic R waves and shortened PR interval, as well as preventing cardiac arrhythmias, such

as premature ventricular contractions (Wehling-Henricks et al. 2005). Thus, increased cardiomyocyte NO levels can improve prominent features of cardiac dystrophic pathology in *mdx* mice.

Not only are increases in NO concentrations cardioprotective, but increases in cGMP concentrations are also cardioprotective in *mdx* hearts (Khairallah et al. 2008). Indeed, cardiomyocyte-specific expression of constitutively active NPR-A (atrial natriuretic peptide receptor A guanylyl cyclase), a plasma membrane-associated guanylyl cyclase, reduced cardiomyopathy in *mdx* hearts (Khairallah et al. 2008). The NPR-A transgene-mediated an increase in cGMP in dystrophin-deficient cardiomyocytes improved cardiomyocyte viability, blunted the progressive increase in LV end diastolic pressure (preload), and increased the cardiac power two-fold, but did not protect *mdx* hearts against contraction-induced damage (Khairallah et al. 2008). These data demonstrated that chronic increases in cardiomyocyte cGMP levels could reduce cardiac dysfunction in *mdx* mice. In summary, as observed in skeletal muscle, several different approaches indicate that enhanced NO-cGMP signaling has potent antidystrophic effects in dystrophin-deficient hearts.

8 Use of PDE5A Inhibitors to Amplify cGMP Signaling in *mdx* Mice

As described above, one of the consequences of NO signaling is an increase in sGC activity, with a concomitant increase in cytosolic cGMP. While it is not possible to selectively increase nNOS expression or activity using a pharmacological approach, it is possible to enhance NO signal transduction by inhibiting the activity of cGMP-hydrolyzing PDEs, such as PDE5A, thus raising cytosolic cGMP. Three studies of the impact of PDE5A inhibition on the dystrophic pathology of cardiac and skeletal muscle in the *mdx* mouse model of DMD have been reported and will now be summarized.

In one published study of the cardiac effects of sildenafil-mediated inhibition of PDE5A in *mdx* mice, Khairallah and coworkers reported that sildenafil administered daily by intraperitoneal injection over 6 weeks (0.7 mg/kg/day) enabled *mdx* hearts to sustain a higher heart rate in response to increased workload (Khairallah et al. 2008). Sildenafil significantly reduced Evan's Blue dye uptake in *mdx* cardiomyocytes, indicative of reduced membrane permeability and suggestive of decreased susceptibility to contraction-induced damage (Khairallah et al. 2008). Sildenafil also decreased *sgcal* ($\alpha 1$ subunit of sGC) and *Anf* (atrial natriuretic factor) transcript expression, suggesting that inhibition of PDE5A improved upstream cGMP signaling and decreased early pathological remodeling in *mdx* hearts, respectively. Cardioprotective effects were not due to utrophin upregulation.

In parallel independent studies, we have found that sildenafil confers significant cardioprotection to old *mdx* hearts (Adamo et al. 2010). Twelve-month-old *mdx* mice exhibit significant LV dysfunction, as indicated by a higher than normal MPI

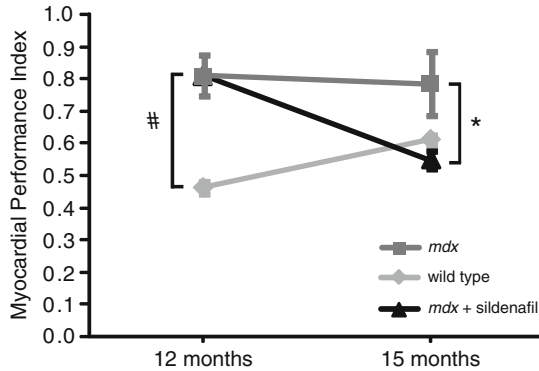


Fig. 2 Sildenafil treatment reverses cardiomyopathy in old *mdx* mice. Twelve-month-old *mdx* mice exhibit significant left ventricle dysfunction as indicated by a high myocardial performance index compared with wild-type mice ($^{\#}p < 0.05$). Twelve-month-old *mdx* mice treated for 3 months with the PDE5 inhibitor sildenafil ad libitum exhibited a significantly reduced ($p < 0.05$) myocardial performance index comparable to wild-type controls. Sildenafil treatment can reverse established global left ventricle dysfunction in old *mdx* mice (Adamo et al. 2010)

measured by echocardiography (Spurney et al. 2008; Adamo et al. 2010, Fig. 2). Treatment of 12-month-old *mdx* mice for 3 months with sildenafil in their drinking water significantly reduces MPI to wild-type levels (Adamo et al. 2010, Fig. 2). Thus, sildenafil can reverse established left ventricle dysfunction, even in aged *mdx* mice. These data suggest that older DMD patients with established cardiomyopathy may benefit from PDE5A inhibition.

Since cardiomyocytes express very little or no PDE5A, the question remains as to how PDE5A inhibition reduces cardiac pathology and dysfunction in the *mdx* heart (Takimoto et al. 2005; Lukowski et al. 2010). PDE5A protein expression may be higher in *mdx* cardiomyocytes, but at present it is unknown whether PDE5A levels or action are affected by the loss of dystrophin. Sildenafil may exert some of its cardioprotective effects by inhibiting PDE5A activity in VSMC of the systemic or cardiac vasculature. In addition, off-target effects of sildenafil on PDE1C could contribute to cardiomyocyte-specific effects of sildenafil. Like PDE5A, PDE1C can hydrolyze cGMP. But unlike PDE5A, PDE1C is found in abundance in cardiomyocytes (Bender and Beavo 2006). High nanomolar concentrations of sildenafil can inhibit PDE1C activity; therefore, inhibition of PDE1C could also account for some of the cardioprotective effects of sildenafil (Lukowski et al. 2010; Vandeput et al. 2009). Nonetheless, it is clear that increasing cGMP levels sildenafil treatment reduces cardiac pathology and dysfunction in *mdx* hearts (Khairallah et al. 2008). On the basis of these findings, Khairallah and coworkers proposed that upregulation of cGMP by PDE5 inhibition should be explored as a new therapeutic approach to treating DMD.

The impact of PDE5 inhibition on contraction-induced muscle damage during ischemic exercise has also been investigated in *mdx* mice (Asai et al. 2007). Restoration of postcontraction blood flow to the sternomastoid muscle in situ was

achieved by acute application of the PDE5 inhibitor, tadalafil. Tadalafil treatment reversed the ischemia and reduced contraction-induced lesions and myofiber death. Pregnant mice were administered tadalafil in their drinking water (1 mg tadalafil per 100 mL); thus, pups had received tadalafil in utero and then also received tadalafil in their drinking water until 4 weeks of age (Asai et al. 2007). Treated hind limb and respiratory skeletal muscles exhibited reduced muscle necrosis and fibrosis (Asai et al. 2007). In addition, treated *mdx* muscles also exhibited decreased variability in myofiber size and a reduction in regenerating centrally nucleated myofibers, suggesting that tadalafil decreased muscle degeneration.

Taken together, these data suggest that tadalafil-mediated inhibition of PDE5A was sufficient to restore blood supply to muscles after exercise and significantly reduced contraction-induced damage in the dystrophin-deficient skeletal muscles. Like the heart, PDE5A inhibition resulted in less contraction-induced damage in *mdx* muscles. The work of Asai and coworkers supports the proposition that vascular therapy with PDE5A inhibitors may be of therapeutic benefit to DMD patients.

Consistent with findings that *mdx* myofibers in situ experience excessive contraction-induced damage under ischemic conditions, *mdx* mice exhibit marked cage inactivity after mild treadmill exercise (Kobayashi et al. 2008). KN1 (nNOS knockout 1) mice also exhibited the same postexercise inactivity, suggesting to the authors that loss of skeletal muscle sarcolemmal nNOS μ was responsible. This postexercise decrease in cage activity is thought to be analogous to the exaggerated fatigue response to mild exercise observed in patients with neuromuscular diseases such as DMD.

To test the impact of improved postcontraction blood flow on postexercise cage activity, Kobayashi and coworkers treated *mdx* and KN1 mice acutely with tadalafil or sildenafil. Sildenafil (300 mg/kg/day) was administered in the food, while tadalafil (300 mg/kg/day) was administered directly by gavage. Both inhibitors were administered the day before and on the day of exercise testing. PDE5A inhibition substantially increased perfusion of the *mdx* hind limb with blood after exercise. Interestingly, postexercise inactivity was reduced 30–40% by PDE5A inhibition in *mdx* mice, but was unaffected in KN1 mice, demonstrating that nNOS expression (nNOS-2, nNOS α , nNOS μ) is required for the postexercise benefits of PDE5A inhibition. This is consistent with findings that NO is required for both basal and maximal activation of sGC activity and for many of the physiological effects of sildenafil (Nagayama et al. 2008; Fernhoff et al. 2009). This may be an important consideration since nNOS levels may be substantially lower or absent in DMD patients, thus lowering the potential efficacy of sildenafil (Chang et al. 1996). Treated *mdx* mice ran twice the distance of untreated *mdx* mice. Despite increased exercise performance, serum CK activity was significantly reduced, suggesting reduced membrane permeability in agreement with results reported by Asai et al. (2007). Together, these data suggest that in dystrophin-deficient muscle tissues, the loss of the vasomodulatory function of nNOS μ may be compensated for, to some degree, by PDE5A inhibition.

The relationship between skeletal muscle performance, blood supply, and nNOS μ is complex. Kobayashi and coworkers concluded that the absence of contraction-induced sarcolemmal nNOS μ signaling to the adjacent VSMC was responsible for postexercise inactivity. While the beneficial effects of PDE5A inhibition are clear, the mechanisms by which they occur are not. Although it remains to be determined whether augmentation of nNOS μ signaling from skeletal to VSMC is the sole pathway responsible for these beneficial effects, it seems unlikely for several reasons. First, augmentation of smooth muscle NO-cGMP signaling alone in *mdx* mice can enhance blood supply and reduce membrane permeability (Ito et al. 2006). Since PDE5A expression is high in VSMC, inhibition of PDE5A may promote smooth muscle relaxation independently of NO produced by skeletal muscle. Second, PDE5A inhibition affects vascular bed function throughout the cardiovascular system and likely enhances systemic hemodynamics during exercise. Third, additional, recently identified nNOS-sGC-PKG signaling pathways in skeletal muscle control postexercise muscle strength that could be affected by PDE5A inhibition in skeletal muscle (Percival et al. 2010). Fourth, nNOS knockout mice exhibit pronounced muscle fatigue and weakness during exercise that could contribute to the observed postexercise weakness (Percival et al. 2008). Finally, acute sildenafil treatment enhances cardiac function in *mdx* mice and could assist cardiovascular recovery (Khairallah et al. 2008). Thus, the factors that contribute to the postexercise inactivity after mild exercise in *mdx* mice are many and likely include muscle weakness during and/or after exercise. The beneficial effects of PDE5A inhibition likely reflect effects at many sites of action, making mechanistic interpretation of these whole animal studies very difficult. Nonetheless, while the molecular mechanisms responsible are debatable, Kobayashi et al. have clearly shown that PDE5A inhibition in *mdx* mice provides nNOS-dependent enhancement of activity during and after exercise as well as reducing exercise-induced muscle damage.

9 Conclusion

Although the examples of therapeutic benefit are few and the mechanisms are poorly understood, the evidence that PDE5A inhibition reduces skeletal and cardiac muscle damage, particularly contraction-induced myofiber damage during exercise, is compelling. PDE5A inhibition also enhances exercise performance, reduces the negative effects of mild exercise, and enhances the workload capacity of dystrophin-deficient hearts. Thus, further studies are required to flesh out this promising therapeutic approach. However, mice are not men and ultimately, any efficacy of PDE5A inhibitors in preclinical studies must be validated in a clinical setting. Efficacy in both skeletal muscle and cardiac tissue makes PDE5A inhibition particularly attractive as a therapeutic approach and warrants further research into the potential utility of PDE5A inhibition in the treatment of cardiovascular disease in DMD.

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References

- Adamo CM, Dai DF, Percival JM, Minami E, Willis MS, Patrucco E, Froehner SC, Beavo JA (2010) Sildenafil reverses cardiac dysfunction in the *mdx* mouse model of Duchenne muscular dystrophy. *Proc Natl Acad Sci USA* 107:19079-19083
- Adams ME, Kramarcy N, Krall SP, Rossi SG, Rotundo RL, Sealock R, Froehner SC (2000) Absence of alpha-syntrophin leads to structurally aberrant neuromuscular synapses deficient in utrophin. *J Cell Biol* 150:1385-1398
- Adams ME, Mueller HA, Froehner SC (2001) In vivo requirement of the alpha-syntrophin PDZ domain for the sarcolemmal localization of nNOS and aquaporin-4. *J Cell Biol* 155:113-122
- Asai A, Sahani N, Kaneki M, Ouchi Y, Martyn JA, Yasuhara SE (2007) Primary role of functional ischemia, quantitative evidence for the two-hit mechanism, and phospho-diesterase-5 inhibitor therapy in mouse muscular dystrophy. *PLoS One* 2:e806
- Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lemmon CA, Burnett AL, O'Rourke B, Rodriguez ER, Huang PL, Lima JA, Berkowitz DE, Hare JM (2002) Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature* 416:337-339
- Bendall JK, Damy T, Ratajczak P, Loyer X, Monceau V, Marty I, Milliez P, Robidel E, Marotte F, Samuel JL, Heymes C (2004) Role of myocardial neuronal nitric oxide synthase-derived nitric oxide in beta-adrenergic hyporesponsiveness after myocardial infarction-induced heart failure in rat. *Circulation* 110:2368-2375
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488-520
- Bia BL, Cassidy PJ, Young ME, Rafael JA, Leighton B, Davies KE, Radda GK, Clarke K (1999) Decreased myocardial nNOS, increased iNOS and abnormal ECGs in mouse models of Duchenne muscular dystrophy. *J Mol Cell Cardiol* 31:1857-1862
- Bloom TJ (2002) Cyclic nucleotide phosphodiesterase isozymes expressed in mouse skeletal muscle. *Can J Physiol Pharmacol* 80:1132-1135
- Bostick B, Yue Y, Long C, Marschalk N, Fine DM, Chen J, Duan D (2009) Cardiac expression of a mini-dystrophin that normalizes skeletal muscle force only partially restores heart function in aged *mdx* mice. *Mol Ther* 17:253-261
- Boulanger CM, Heymes C, Benessiano J, Geske RS, Lévy BI, Vanhoutte PM (1998) Neuronal nitric oxide synthase is expressed in rat vascular smooth muscle cells: activation by angiotensin II in hypertension. *Circ Res* 83:1271-1278
- Bredt DS, Snyder SH (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci USA* 87:682-685
- Brenman JE, Chao DS, Xia H, Aldape K, Bredt DS (1995) Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82:743-752
- Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Froehner SC, Bredt DS (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* 84:757-767
- Bushby K, Finkel R, Birnkrant DJ, Case LE, Clemens PR, Cripe L, Kaul A, Kinnett K, McDonald C, Pandya S, Poysky J, Shapiro F, Tomezsko J, Constantin C, DMD Care Considerations Working Group (2009) Diagnosis and management of Duchenne muscular

- dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *Lancet Neurol* 9:77–93
- Chamberlain JS, Metzger J, Reyes M, Townsend D, Faulkner JA (2007) Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. *FASEB J* 21:2195–2204
- Chang WJ, Iannaccone ST, Lau KS, Masters BS, McCabe TJ, McMillan K, Padre RC, Spencer MJ, Tidball JG, Stull JT (1996) Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy. *Proc Natl Acad Sci USA* 93:9142–9147
- Chao DS, Gorospe JR, Brenman JE, Rafael JA, Peters MF, Froehner SC, Hoffman EP, Chamberlain JS, Bredt DS (1996) Selective loss of sarcolemmal nitric oxide synthase in Becker muscular dystrophy. *J Exp Med* 184:609–618
- Chao DS, Silvagno F, Xia H, Cornwell TL, Lincoln TM, Bredt DS (1997) Nitric oxide synthase and cyclic GMP-dependent protein kinase concentrated at the neuromuscular endplate. *Neuroscience* 76:665–672
- Chenard AA, Becane HM, Tertrain F, de Kermadec JM, Weiss YA (1993) Ventricular arrhythmia in Duchenne muscular dystrophy: prevalence, significance and prognosis. *Neuromuscul Disord* 3:201–206
- Chu V, Otero JM, Lopez O, Sullivan MF, Morgan JP, Amende I, Hampton TG (2002) Electrocardiographic findings in mdx mice: a cardiac phenotype of Duchenne muscular dystrophy. *Muscle Nerve* 26:513–519
- Colussi C, Mozzetta C, Gurtner A, Illi B, Rosati J, Straino S, Ragone G, Pescatori M, Zaccagnini G, Antonini A, Minetti G, Martelli F, Piaggio G, Gallinari P, Steinkuhler C, Clementi E, Dell'Aversana C, Altucci L, Mai A, Capogrossi MC, Puri PL, Gaetano C (2008) HDAC2 blockade by nitric oxide and histone deacetylase inhibitors reveals a common target in Duchenne muscular dystrophy treatment. *Proc Natl Acad Sci USA* 105:19183–19187
- Craven KB, Zagotta WN (2006) CNG and HCN channels: two peas, one pod. *Annu Rev Physiol* 68:375–401
- Crosbie RH, Barresi R, Campbell KP (2002) Loss of sarcolemma nNOS in sarcoglycan-deficient muscle. *FASEB J* 16:1786–1791
- Danielou G, Comtois AS, Dudley R, Karpati G, Vincent G, Des Rosiers C, Petrof BJ (2001) Dystrophin-deficient cardiomyocytes are abnormally vulnerable to mechanical stress-induced contractile failure and injury. *FASEB J* 15:1655–1667
- Das A, Xi L, Kukreja RC (2008) Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. *J Biol Chem* 283:29572–29585
- Davies KE, Nowak KJ (2006) Molecular mechanisms of muscular dystrophies: old and new players. *Nat Rev Mol Cell Biol* 7:762–773
- de Kermadec JM, Bécane HM, Chénard A, Tertrain F, Weiss Y (1994) Prevalence of left ventricular systolic dysfunction in Duchenne muscular dystrophy: an echocardiographic study. *Am Heart J* 127:618–623
- Dellorusso C, Crawford RW, Chamberlain JS, Brooks SV (2001) Tibialis anterior muscles in mdx mice are highly susceptible to contraction-induced injury. *J Muscle Res Cell Motil* 22:467–475
- Eagle M, Bourke J, Bullock R, Gibson M, Mehta J, Giddings D, Straub V, Bushby K (2007) Managing Duchenne muscular dystrophy—the additive effect of spinal surgery and home nocturnal ventilation in improving survival. *Neuromuscul Disord* 17:470–475
- Ervasti JM (2007) Dystrophin, its interactions with other proteins, and implications for muscular dystrophy. *Biochim Biophys Acta* 1772:108–117
- Ervasti JM, Campbell KP (1993) Dystrophin and the membrane skeleton. *Curr Opin Cell Biol* 5:82–87
- Eu JP, Sun J, Xu L, Stamler JS, Meissner G (2000) The skeletal muscle calcium release channel: coupled O₂ sensor and NO signaling functions. *Cell* 102:499–509
- Fernhoff NB, Derbyshire ER, Marletta MA (2009) A nitric oxide/cysteine interaction mediates the activation of soluble guanylate cyclase. *Proc Natl Acad Sci USA* 106:21602–21607

- Finsterer J, Stöllberger C (2003) The heart in human dystrophinopathies. *Cardiology* 99:1–19
- Firestein BL, Bredt DS (1999) Interaction of neuronal nitric-oxide synthase and phosphofructokinase-M. *J Biol Chem* 274:10545–10550
- Frankel KA, Rosser RJ (1976) The pathology of the heart in progressive muscular dystrophy: epimyocardial fibrosis. *Hum Pathol* 7:375–386
- Hillier BJ, Christopherson KS, Prehoda KE, Bredt DS, Lim WA (1999) Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex. *Science* 284:812–815
- Hoffman EP, Brown RH Jr, Kunkel LM (1987) Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell* 51:919–928
- Hofmann F, Bernhard D, Lukowski R, Weinmeister P (2009) cGMP regulated protein kinases (cGK). *Handb Exp Pharmacol* 191:137–162
- Ishikawa Y, Bach JR, Minami R (1999) Cardioprotection for Duchenne's muscular dystrophy. *Am Heart J* 137:895–902
- Ito K, Kimura S, Ozasa S, Matsukura M, Ikezawa M, Yoshioka K, Ueno H, Suzuki M, Araki K, Yamamura K, Miwa T, Dickson G, Thomas GD, Miike T (2006) Smooth muscle-specific dystrophin expression improves aberrant vasoregulation in mdx mice. *Hum Mol Genet* 15:2266–2275
- Jerusalem F, Engel AG, Gomez MR (1974) Duchenne dystrophy I. morphometric study of the muscle microvasculature. *Brain* 97:115–122
- Kameya S, Miyagoe Y, Nonaka I, Ikemoto T, Endo M, Hanaoka K, Nabeshima Y, Takeda S (1999) alpha1-syntrophin gene disruption results in the absence of neuronal-type nitric-oxide synthase at the sarcolemma but does not induce muscle degeneration. *J Biol Chem* 274:2193–2200
- Khairallah M, Khairallah R, Young ME, Dyck JR, Petrof BJ, Des Rosiers C (2007) Metabolic and signaling alterations in dystrophin-deficient hearts precede overt cardiomyopathy. *J Mol Cell Cardiol* 43:119–1129
- Khairallah M, Khairallah RJ, Young ME, Allen BG, Gillis MA, Danialou G, Deschepper CF, Petrof BJ, Des Rosiers C (2008) Sildenafil and cardiomyocyte-specific cGMP signaling prevent cardiomyopathic changes associated with dystrophin deficiency. *Proc Natl Acad Sci USA* 105:7028–7033
- Kobayashi YM, Rader EP, Crawford RW, Iyengar NK, Thedens DR, Faulkner JA, Parikh SV, Weiss RM, Chamberlain JS, Moore SA, Campbell KP (2008) Sarcolemma-localized nNOS is required to maintain activity after mild exercise. *Nature* 456:511–515
- Koenig M, Monaco AP, Kunkel LM (1988) The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* 53:219–228
- Lai Y, Thomas GD, Yue Y, Yang HT, Li D, Long C, Judge L, Bostick B, Chamberlain JS, Terjung RL, Duan D (2009) Dystrophins carrying spectrin-like repeats 16 and 17 anchor nNOS to the sarcolemma and enhance exercise performance in a mouse model of muscular dystrophy. *J Clin Invest* 119:624–635
- Lukowski R, Rybalkin SD, Loga F, Leiss V, Beavo JA, Hofmann F (2010) Cardiac hypertrophy is not amplified by deletion of cGMP-dependent kinase-I in cardiomyocytes. *Proc Natl Acad Sci USA* 107:5646–5651
- Manzur AY, Kuntzer T, Pike M, Swan A (2008) Glucocorticoid corticosteroids for Duchenne muscular dystrophy. *Cochrane Database Syst Rev* 1:CD003725
- Markham LW, Michelfelder EC, Border WL, Khoury PR, Spicer RL, Wong BL, Benson DW, Cripe LH (2006) Abnormalities of diastolic function precede dilated cardiomyopathy associated with Duchenne muscular dystrophy. *J Am Soc Echocardiogr* 19:865–871
- McConnell GK, Wadley GD (2008) Potential role of nitric oxide in contraction-stimulated glucose uptake and mitochondrial biogenesis in skeletal muscle. *Clin Exp Pharmacol Physiol* 35:1488–1492
- McConnell GK, Bradley SJ, Stephens TJ, Canny BJ, Kingwell BA, Lee-Young RS (2007) Skeletal muscle nNOS mu protein content is increased by exercise training in humans. *Am J Physiol Regul Integr Comp Physiol* 293:R821–R828

- Mendell JR, Engel WK, Derrer EC (1971) Duchenne muscular dystrophy: functional ischemia reproduces its characteristic lesions. *Science* 172:1143–1145
- Mergia E, Koelsing D, Friebe A (2009) Genetic mouse models of the NO receptor 'soluble' guanylyl cyclases. *Handb Exp Pharmacol* 191:33–46
- Moriuchi T, Kagawa N, Mukoyama M, Hizawa K (1993) Autopsy analyses of the muscular dystrophies Tokushima. *J Exp Med* 40:83–93
- Nagayama T, Zhang M, Hsu S, Takimoto E, Kass DA (2008) Sustained soluble guanylate cyclase stimulation offsets nitric-oxide synthase inhibition to restore acute cardiac modulation by sildenafil. *J Pharmacol Exp Ther* 326:380–387
- Percival JM, Adams ME, Froehner SC (2006) Syntrophin: a molecular adaptor conferring a signaling role to the dystrophin-associated protein complex. In: Winder SJ (ed) *Molecular mechanisms of muscular dystrophies*. Landes Bioscience, TX
- Percival JM, Anderson KN, Gregorevic P, Chamberlain JS, Froehner SC (2008) Functional deficits in nNOSmu-deficient skeletal muscle: myopathy in nNOS knockout mice. *PLoS ONE* 3:e3387
- Percival JM, Anderson KN, Huang PL, Adams ME, Froehner SC (2010) Golgi and sarcolemmal nNOS isozymes differentially regulate contraction-induced fatigue and vasoconstriction in exercising skeletal muscle. *J Clin Invest* 120:816–826
- Petrof BJ, Shrager JB, Stedman HH, Kelly AM, Sweeney HL (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc Natl Acad Sci USA* 90:3710–3714
- Quinlan JG, Hahn HS, Wong BL, Lorenz JN, Wenisch AS, Levin LS (2004) Evolution of the mdx mouse cardiomyopathy: physiological and morphological findings. *Neuromuscul Disord* 14:491–496
- Reffellmann T, Kloner RA (2009) Phosphodiesterase 5 inhibitors: are they cardioprotective? *Cardiovasc Res* 83:204–12
- Sander M, Chavoshan B, Harris SA, Iannaccone ST, Stull JT, Thomas GD, Victor RG (2000) Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. *Proc Natl Acad Sci USA* 97:13818–13823
- Saraiva RM, Minhas KM, Raju SV, Barouch LA, Pitz E, Schuleri KH, Vandegaer K, Li D, Hare JM (2005) Deficiency of neuronal nitric oxide synthase increases mortality and cardiac remodeling after myocardial infarction: role of nitroso-redox equilibrium. *Circulation* 112:3415–3422
- Sears CE, Bryant SM, Ashley EA, Lygate CA, Rakovic S, Wallis HL, Neubauer S, Terrar DA, Casadei B (2003) Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. *Circ Res* 92:e52–e59
- Seddon M, Melikian N, Dworakowski R, Shabeeh H, Jiang B, Byrne J, Casadei B, Chowiecnyk P, Shah AM (2009) Effects of neuronal nitric oxide synthase on human coronary artery diameter and blood flow in vivo. *Circulation* 119:2656–2662
- Senzaki H, Smith CJ, Juang GJ, Isoda T, Mayer SP, Ohler A, Paolocci N, Tomaselli GF, Hare JM, Kass DA (2001) Cardiac phosphodiesterase 5 (cGMP-specific) modulates beta-adrenergic signaling in vivo and is down-regulated in heart failure. *FASEB J* 15:1718–1726
- Silvagno F, Xia H, Bredt DS (1996) Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J Biol Chem* 271:11204–11208
- Spurney CF, Knoblach S, Pistilli EE, Nagaraju K, Martin GR, Hoffman EP (2008) Dystrophin-deficient cardiomyopathy in mouse: expression of Nox4 and Lox are associated with fibrosis and altered functional parameters in the heart. *Neuromuscul Disord* 18:371–381
- Stedman HH, Sweeney HL, Shrager JB, Maguire HC, Panettieri RA, Petrof B, Narusawa M, Leferovich JM, Sladky JT, Kelly AM (1991) The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* 352:536–539
- Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S (2004) Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem* 279:36167–36170
- Suzuki N, Motohashi N, Uezumi A, Fukada S, Yoshimura T, Itoyama Y, Aoki M, Miyagoe-Suzuki Y, Takeda S (2007) NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS. *J Clin Invest* 117:2468–2476

- Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, Bedja D, Gabrielson KL, Wang Y, Kass DA (2005) Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med* 11:214–222
- Thomas GD, Victor RG (1998) Nitric oxide mediates contraction-induced attenuation of sympathetic vasoconstriction in rat skeletal muscle. *J Physiol* 506:817–826
- Thomas GD, Sander M, Lau KS, Huang PL, Stull JT, Victor RG (1998) Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle. *Proc Natl Acad Sci USA* 95:15090–15095
- Thomas GD, Shaul PW, Yuhanna IS, Froehner SC, Adams ME (2003) Vasomodulation by skeletal muscle-derived nitric oxide requires alpha-syntrophin-mediated sarcolemmal localization of neuronal nitric oxide synthase. *Circ Res* 92:554–560
- Thrush PT, Allen HD, Viollet L, Mendell JR (2009) Re-examination of the electrocardiogram in boys with Duchenne muscular dystrophy and correlation with its dilated cardiomyopathy. *Am J Cardiol* 103:262–265
- Tidball JG, Wehling-Henricks M (2004) Expression of a NOS transgene in dystrophin-deficient muscle reduces muscle membrane damage without increasing the expression of membrane-associated cytoskeletal proteins. *Mol Genet Metab* 82:312–320
- Townsend D, Yasuda S, Li S, Chamberlain JS, Metzger JM (2008) Emergent dilated cardiomyopathy caused by targeted repair of dystrophic skeletal muscle. *Mol Ther* 16:832–835
- Vandebrouck C, Dupont G, Cognard C, Raymond G (2001) Cationic channels in normal and dystrophic human myotubes. *Neuromuscul Disord* 11:72–79
- Vandeput F, Krall J, Ockaili R, Salloum FN, Florio V, Corbin JD, Francis SH, Kukreja RC, Movsesian MA (2009) cGMP-hydrolytic activity and its inhibition by sildenafil in normal and failing human and mouse myocardium. *J Pharmacol Exp Ther* 330:884–891
- Wallis RM, Corbin JD, Francis SH, Ellis P (1999) Tissue distribution of phosphodiesterase families and the effects of sildenafil on tissue cyclic nucleotides, platelet function, and the contractile responses of trabeculae carneae and aortic rings in vitro. *Am J Cardiol* 83:3C–12C
- Ward ME, Toporsian M, Scott JA, Teoh H, Govindaraju V, Quan A, Wener AD, Wang G, Bevan SC, Newton DC, Marsden PA (2005) Hypoxia induces a functionally significant and translationally efficient neuronal NO synthase mRNA variant. *J Clin Invest* 115:3128–3139
- Wehling M, Spencer MJ, Tidball JG (2001) A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. *J Cell Biol* 155:123–131
- Wehling-Henricks M, Jordan MC, Roos KP, Deng B, Tidball JG (2005) Cardiomyopathy in dystrophin-deficient hearts is prevented by expression of a neuronal nitric oxide synthase transgene in the myocardium. *Hum Mol Genet* 14:1921–1933
- Wehling-Henricks M, Oltmann M, Rinaldi C, Myung KH, Tidball JG (2009) Loss of positive allosteric interactions between neuronal nitric oxide synthase and phosphoructokinase contributes to defects in glycolysis and increased fatigability in muscular dystrophy. *Hum Mol Genet* 18:3439–3451
- Whitehead NP, Streamer M, Lusambili LI, Sachs F, Allen DG (2006) Streptomycin reduces stretch-induced membrane permeability in muscles from mdx mice. *Neuromuscul Disord* 16:845–854
- Willmann R, Possekkel S, Dubach-Powell J, Meier T, Ruegg MA (2009) Mammalian animal models for Duchenne muscular dystrophy. *Neuromuscul Disord* 19:241–249
- Wu B, Moulton HM, Iversen PL, Jiang J, Li J, Li J, Spurney CF, Sali A, Guerrero AD, Nagaraju K, Doran T, Lu P, Xiao X, Lu QL (2008) Effective rescue of dystrophin improves cardiac function in dystrophin-deficient mice by a modified morpholino oligomer. *Proc Natl Acad Sci USA* 105:14814–14819
- Xu KY, Huso DL, Dawson TM, Bredt DS, Becker LC (1999) Nitric oxide synthase in cardiac sarcoplasmic reticulum. *Proc Natl Acad Sci USA* 96:657–662
- Zhang YH, Zhang MH, Sears CE, Emanuel K, Redwood C, El-Armouche A, Kranias EG, Casadei B (2008) Reduced phospholamban phosphorylation is associated with impaired relaxation in left ventricular myocytes from neuronal NO synthase-deficient mice. *Circ Res* 102:242–249

Phosphodiesterases as Targets for Modulating T-Cell Responses

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Abstract The cAMP–protein kinase A (PKA) signaling pathway is strongly involved in the regulation and modulation of immune responses, and cAMP is the most potent and acute inhibitor of T-cell activation. Thus, cAMP levels in the cell must be tightly regulated. Cyclic AMP-specific phosphodiesterases (PDEs) provide the only mechanism for degrading cAMP in cells, thereby functioning as key regulators of signaling. To obtain a complete immune response with optimal cytokine production and T-cell proliferation, ligation of both the T-cell receptor (TCR) and the CD28 receptor is required. However, engagement of the TCR in primary T cells is followed by rapid cAMP production in lipid rafts and activation of the cAMP–PKA–Csk pathway inhibiting proximal T-cell signaling. In contrast, TCR/CD28 costimulation leads to the recruitment of a PDE4/ β -arrestin complex to rafts in a phosphatidylinositol 3-kinase (PI3K)-dependent manner, resulting in the down-regulation of cAMP levels. Thus, the activities of both PKA and PDE4 seem to be important for regulation of TCR-induced signaling and T-cell function. The use of selective inhibitors has revealed that PDEs are important drug targets in several diseases with an inflammatory component where immune function is important such as asthma, chronic obstructive pulmonary disease (COPD), cardiovascular

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diseases, and neurological disorders. PDEs are also interesting drug targets in immunosuppression following transplantation and for modulation of immune responses.

Keywords cAMP · CD28 · TCR · T-cell function

1 Regulation of cAMP Levels in T Cells

The common and versatile second messenger cAMP controls numerous cellular processes and is known to be a potent inhibitor of T-cell proliferation and cytokine production (Aandahl et al. 2002; Skalhegg et al. 1992). Cyclic AMP can activate protein kinase A (PKA) (Walsh et al. 1968), Epac (exchange protein directly activated by cAMP) (de Rooij et al. 1998; Kawasaki et al. 1998), and cAMP-regulated ion channels (Kaupp and Seifert 2002; Matulef and Zagotta 2003). However, effects mediated by cAMP in T cells are most likely a result of PKA activation as Epac and cAMP-gated channels are absent from or appear to be expressed at low levels in T lymphocytes (our unpublished data and Kawasaki et al. 1998). The cAMP–PKA signaling pathway in T cells is initiated by binding of prostaglandin E₂ (PGE₂) and other extracellular ligands such as catecholamines, serotonin, adenosine, and histamine to G protein-coupled receptors (GPCRs), activation of adenylyl cyclase (AC), and the subsequent generation of cAMP from ATP (Hanoune and Defer 2001). Endogenous cAMP levels in T cells are also influenced by other mechanisms such as those described in the following section involving regulatory T cells (Tregs).

Human Treg cells comprise 5–10% of the peripheral CD4⁺ T-cell population (Ng et al. 2001) and are functionally characterized by their ability to suppress effector T cells in addition to having an essential role in maintaining immunological tolerance (Sakaguchi et al. 2001, 2006; Sakaguchi 2004). This subset of CD4⁺ T cells has been under intense research in the last decade due to its role in various clinical conditions such as autoimmune disease, cancer, and chronic viral infections (Beyer and Schultze 2006; Rouse et al. 2006; Sakaguchi et al. 2001). Tregs can further be subdivided into naturally occurring Tregs that are derived in the thymus and adaptive or peripherally induced Tregs that are developed from naïve T cells in the periphery during immune activation (Bluestone and Abbas 2003). The role of cAMP in induction of Tregs and Treg-mediated suppression is getting increasing attention (reviewed in Yaqub and Tasken 2008). Naturally occurring Tregs have been shown to have high cAMP levels and to mediate their suppressive function by transferring cAMP to effector T cells through gap junctions (Bopp et al. 2007). The high cAMP levels in Tregs can at least partly be explained by the FOXP3-dependent downregulation of phosphodiesterase 3B (PDE3B) (Gavin et al. 2007). Recently, it was also demonstrated that stimulation of mouse Tregs by IL-2 strongly induced adenylyl cyclase 7 (AC7) activity, resulting in cAMP accumulation (Bazhin et al. 2010). Furthermore, the ecto-enzymes CD39 and CD73 expressed on naturally

occurring Tregs may degrade ATP and catalyze the generation of adenosine that acts via the adenosine A2a receptor on activated effector T cells, thereby suppressing their function by intracellular production of cAMP (Deaglio et al. 2007). In addition, continuous antigen exposure to T cells leads to generation of adaptive Tregs (Aandahl et al. 2004). Adaptive Tregs express cyclooxygenase 2 (COX-2), leading to secretion of PGE₂. PGE₂ stimulates FOXP3 expression in Tregs in addition to inhibit effector T-cell function through activation of the cAMP–PKA–Csk signaling pathway (Mahic et al. 2006). The different mechanisms affecting effector T cells described above are schematically summarized in Fig. 1.

The spatiotemporal regulation of cAMP levels within the cell is a result of the combined action of ACs and phosphodiesterases (PDEs), which provide the only mechanism for cAMP degradation (Conti and Jin 1999; Houslay and Adams 2003). Specifically, compartmentalization of receptors, cyclases, and PKA by A-kinase-anchoring proteins (AKAPs) (Michel and Scott 2002) in addition to generation of local pools of cAMP within the cell by the action of PDEs (Zaccolo and Pozzan 2002) results in a high degree of specificity in PKA-mediated signaling, although there are multiple receptors and targets all using cAMP as a second messenger.

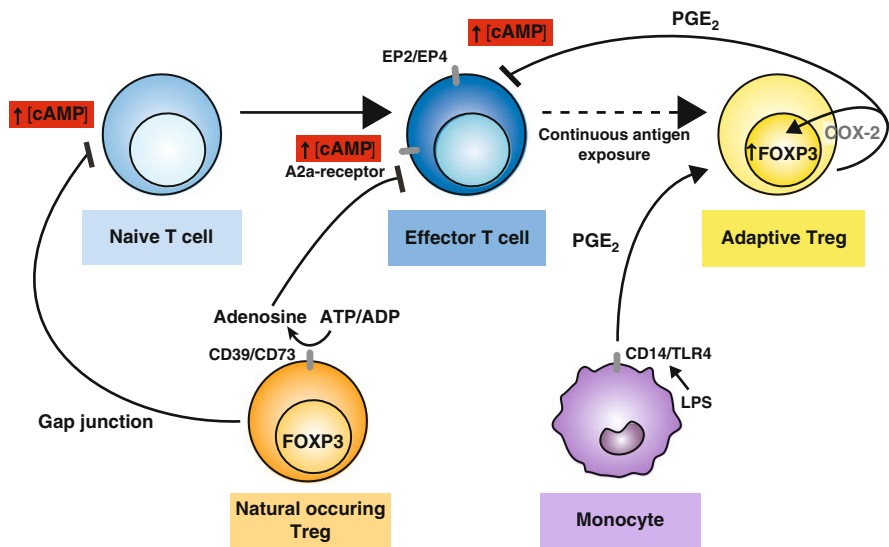


Fig. 1 Schematic model of cAMP-mediated induction of Treg cells and suppression of effector T cells. Naturally occurring Tregs have high cAMP levels and mediate their suppressive function by transferring cAMP to responder T cells through gap junctions. In addition, the ecto-enzymes CD39 and CD73 expressed on naturally occurring Tregs degrade ATP/ADP and catalyze the generation of adenosine, acting via the adenosine A2a receptor on activated effector T cells and thereby suppressing their function by intracellular production of cAMP. Furthermore, continuous activation of T cells results in generation of adaptive Tregs that express COX-2, leading to PGE₂ production. Increased PGE₂ levels stimulate further FOXP3 expression in Treg cells in addition to inhibiting effector T-cell function. Finally, T-cell inhibition and induction of FOXP3 due to PGE₂ secretion can also be a result of LPS-activated monocytes

Furthermore, AKAPs also contribute to versatility by assembling multiprotein signal complexes allowing signal termination by phosphoprotein phosphatases and crosstalk between different signaling pathways (Michel and Scott 2002; Tasken and Aandahl 2004). More specifically, integrating PDEs into these anchoring complexes provide an efficient way to tightly regulate cAMP action in specific cellular environments (reviewed in Baillie et al. 2005; Smith and Scott 2002).

1.1 The cAMP–PKA–Csk Inhibitory Pathway Modulates T-Cell Immune Function

Proteins involved in proximal T-cell receptor (TCR) signaling are localized in lipid rafts, membrane microdomains enriched in cholesterol and sphingolipids (Montixi et al. 1998; Xavier et al. 1998) where they guide signal transduction through formation of a diversity of signaling complexes (Jordan et al. 2003). The multimeric TCR complex is composed of two functional units: one involved in ligand binding ($\alpha\beta$) and the other in signal transduction (CD3 γ , δ , ϵ , and ζ chains). Upon TCR engagement one of the most proximal events taking place in T cells is activation of the Src family protein tyrosine kinases (SFKs), in particular Lck. The following phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) present in the CD3 subunits results in recruitment of the tandem SH2-containing tyrosine kinase ZAP-70. ZAP-70 becomes fully active upon Lck-mediated phosphorylation and plays an essential role with respect to further propagation of the signal downstream of the TCR (Zhang et al. 1998). A complete immune response finally leads to activation of the transcription factors nuclear factor κ B (NF- κ B) and nuclear factor of activated T cells (NFAT) initiating cytokine production and T-cell proliferation (reviewed in Rudd 1999). To obtain proper T-cell activation and to prevent activation-induced cell death, tight regulation of the initial key enzyme Lck is required.

Phosphorylation of a conserved tyrosine residue (Y394) in the catalytic domain of Lck gives rise to a conformational change in the activation loop of the enzyme, thereby allowing substrate binding and catalysis to take place (Yamaguchi and Hendrickson 1996). In contrast, Lck is negatively regulated by phosphorylation of a conserved C-terminal tyrosine residue (Y505) by C-terminal Src kinase (Csk), thereby inducing an intramolecular interaction between this phospho site and the Lck SH2 domain (Bergman et al. 1992; Okada and Nakagawa 1989). In resting T cells, Csk is constitutively localized in proximity to its substrates Lck and Fyn through docking to the hyperphosphorylated transmembrane adaptor protein Cbp/PAG in lipid rafts (Brdicka et al. 2000; Kawabuchi et al. 2000). However, upon T-cell activation, Cbp/PAG is rapidly dephosphorylated by CD45, and Csk is transiently displaced to the cytosol (Brdicka et al. 2000; Davidson et al. 2003; Torgersen et al. 2001). Fyn-mediated phosphorylation of Cbp/PAG results in rerecruitment of Csk and reestablishment of the inhibitory pathway (Brdicka et al. 2000; Kawabuchi et al. 2000). Docking of Csk to Cbp/PAG in addition to phosphorylation by PKA

increases Csk activity six- to eightfold and potently inhibits TCR-dependent activation of Lck (Vang et al. 2001, 2003). Furthermore, both Csk and PKA are located to lipid rafts through anchoring to a Cbp/PAG–EBP50–Ezrin scaffold and PKA is thus positioned for precise spatiotemporal regulation of Csk (Ruppelt et al. 2007). In conclusion, cAMP inhibits TCR-induced T-cell activation and exerts important immunoregulatory functions through a cAMP–PKA–Csk inhibitory pathway assembled in lipid rafts and acting on the Src family tyrosine kinase Lck (reviewed in Torgersen et al. 2002).

1.2 PDEs Are Key Regulatory Players in Modulating T-Cell Immune Responses

PDEs provide the only mechanism for degrading cAMP in cells and function as important regulators of signaling. The recent realization that PDEs play a key role in compartmentalized cAMP signaling may at least partly explain why there are so many PDE isoforms encoded by mammalian genomes. The PDE superfamily currently consists of 11 members and more than 50 isoforms that are distributed in different tissues and at varying levels due to multiple genes within each family in addition to the use of distinct promoters and mRNA splicing (reviewed in Conti and Beavo 2007). PDEs share a common structural organization with a conserved catalytic domain flanked by regulatory domains both in the N- and C-terminus. These family-specific regulatory domains include phosphorylation sites, membrane targeting domains, dimerization domains, and binding sites for small ligands (reviewed in Houslay 2009; Mehats et al. 2002). Most cells express several different PDEs and the cellular expression differs between different cell types, suggesting that each PDE exerts a specialized function. Studies using PDE-deficient mice (Hansen et al. 2000; Jin et al. 1999, 2005; Jin and Conti 2002; Masciarelli et al. 2004; Yang et al. 2003), siRNA-mediated PDE knockdown (Lynch et al. 2005), and dominant-negative PDEs (Baillie et al. 2003; Lynch et al. 2005; McCahill et al. 2005) have indeed demonstrated that particular isoforms have unique, nonredundant functional roles. Furthermore, the use of selective inhibitors has revealed that PDEs are important drug targets in several diseases such as asthma, chronic obstructive pulmonary disease (COPD), cardiovascular diseases, and neurological and psychiatric disorders such as schizophrenia and erectile dysfunctions (Corbin et al. 2002; Feldman and McNamara 2002; Houslay et al. 2005; Manji et al. 2003; Millar et al. 2005; Spina 2003). PDEs are also interesting drug targets for immunomodulation since cAMP as mentioned above inhibits immune functions. In addition, crystal structures of different PDE catalytic domains in complex with substrate products or inhibitors have recently been solved, and this information is critical for the development of inhibitors with increased potency and selectivity (reviewed in Ke and Wang 2007). The PDE5-specific inhibitors sildenafil (Viagra™), vardenafil (Levitra™), and tadalafil (Cialis™) are examples of selective inhibitors

that have been successfully used for treatment of male erectile dysfunction (Francis and Corbin 2005) and have also been approved for the treatment of pulmonary hypertension (reviewed in Vlachopoulos et al. 2009). PDE4-selective inhibitors have shown potential for treatment of asthma and COPD, whereas the PDE3-specific inhibitors have been approved for treatment of intermittent claudication (reviewed in Conti and Beavo 2007; Houslay et al. 2005; Spina 2008; Thompson et al. 2007).

In addition to PDE3, PDE4, and PDE7, T cells also express PDE1, PDE2, PDE5, and PDE8 (reviewed in Essayan 2001). However, isoforms from the PDE4 subfamily seem to represent the majority of cAMP hydrolyzing activity in T cells (Erdogan and Houslay 1997; Giembycz et al. 1996). PDE4 isoforms are highly conserved through evolution and four genes (PDE4A/B/C/D) encode over 20 distinct isoforms (reviewed in Conti and Beavo 2007; Houslay et al. 2007; Houslay and Adams 2003). The PDE genes have complex structures including several intronic promoters. In addition to alternate splicing, these promoters give rise to a number of alternately initiated transcripts. The various PDE4 proteins are characterized by a unique N-terminal region followed by the presence of upstream conserved regions UPCR1 and/or UPCR2, resulting in long, short, and supershort PDE4 isoforms. It is now becoming clear that these unique regions are important in intracellular targeting as well as for functional significance of individual isoforms (reviewed in Conti and Beavo 2007; Houslay et al. 2007). Furthermore, an increasing list of proteins have recently been shown to interact directly with various PDE4 isoforms, including scaffolding proteins such as AKAPs, β -arrestin, and RACK1 and kinases such as Erk and Src family tyrosine kinases (reviewed in Houslay 2009). These diverse lists of interaction partners that sequester PDE4 isoforms clearly indicate that targeting of these enzymes is very important. However, relatively little is still known about the precise function of the different PDE4 isoforms, although PDE4B1 has been linked to schizophrenia (Millar et al. 2005), PDE4D4 to prostate cancer (Prins et al. 2008), and PDE4D7 to stroke (Gretarsdottir et al. 2003). PDE4 isoforms in complex with β -arrestin have recently been shown to have an important role in T-cell activation and this is discussed in detail below.

1.3 TCR-Induced cAMP Production in T-Cell Lipid Rafts

TCR ligation has previously been demonstrated to induce cAMP production in the cell (Ledbetter et al. 1986). However, since increased cAMP concentrations inhibit T-cell function and proliferation (Aandahl et al. 2002; Skalhegg et al. 1992), TCR-mediated cAMP production must be tightly regulated. The significance of activation-induced cAMP production has still not been fully understood and the spatiotemporal dynamics of the activation-induced cAMP gradient has not been completely appreciated. However, we were able to show that upon engagement of the TCR in primary T cells, cAMP was rapidly produced in lipid rafts (Abrahamsen et al. 2004). This activates a pool of PKA targeted to rafts by association with the

anchoring protein Ezrin, forming part of a multimolecular complex where Ezrin, EBP50, and Cbp/PAG provide a scaffold able to coordinate PKA phosphorylation and activation of Csk, thereby inhibiting T-cell activation (Ruppelt et al. 2007; Vang et al. 2001). It has been shown that G_i , G_s , and AC segregate into lipid rafts (Oh and Schnitzer 2001), and our data indicate that raft recruitment of the stimulatory G-protein G_s and dissociation of the inhibitory G-protein G_i play an important role in the cAMP production that occurs after TCR ligation. A local increase in cAMP is, therefore, generated in T-cell lipid rafts upon TCR stimulation alone. In contrast, TCR/CD28 costimulated T cells revealed decreased cAMP levels compared to control cells and an increase in cAMP levels was only observed in the presence of the nonselective PDE inhibitor IBMX. Furthermore, PKA substrates in lipid rafts were rapidly phosphorylated in cells only activated through the TCR (Abrahamsen et al. 2004). In conclusion, this suggests that TCR-induced cAMP production constitutes a negative feedback loop in the absence of a second stimulatory signal and that this inhibition can only be lifted through cAMP degradation by PDEs, thus allowing a complete T-cell activation to proceed (see Fig. 2 for an overview).

1.3.1 PDE4 is Recruited to T-Cell Lipid Rafts Upon TCR/CD28 Costimulation

As already mentioned, isoforms from the PDE4 subfamily account for the majority of the cAMP hydrolyzing activity in T cells (Erdogan and Houslay 1997; Giembycz et al. 1996), and in accordance with this, we have observed PDE4 activity in lipid rafts upon T-cell activation. In contrast, no PDE3 activity was detected (Abrahamsen et al. 2004). In particular, TCR/CD28 costimulation resulted in a rapid recruitment of PDE4 activity to lipid rafts, indicating that temporal changes in PDE4 activity can play a key role in tuning intracellular activation-induced gradients of cAMP in T-cell lipid rafts and thereby increase signal propagation upon costimulation (Abrahamsen et al. 2004). The mechanisms by which PDE4 isoforms are recruited to specific locations upon T-cell activation are now being unravelled (Abrahamsen et al. 2004; Arp et al. 2003; Bjorgo et al. 2010). PDE4B has been reported to associate with the TCR complex (Baroja et al. 1999) and transfected PDE4B has been shown to relocate to the synapse area between the Jurkat T cell and the antigen-presenting cell (APC) upon contact (Arp et al. 2003). We have demonstrated that PDE4A4, PDE4B2, and PDE4D1/2 are recruited to lipid rafts upon TCR/CD28 costimulation in human primary T cells (Abrahamsen et al. 2004).

Spatial organization and recruitment of mediators of specific pathways are essential to ensure both signaling specificity and amplification. The scaffolding β -arrestin proteins have been reported to confer crosstalk with a growing list of molecules important in cellular trafficking and signal transduction, including Src family protein tyrosine and MAP kinases (reviewed in DeWire et al. 2007). Members of the PDE4 family have previously been described to associate with β -arrestin, and this scaffolding protein has been shown to be responsible for bringing PDE4 to the plasma membrane of HEK293 (Bolger et al. 2003;

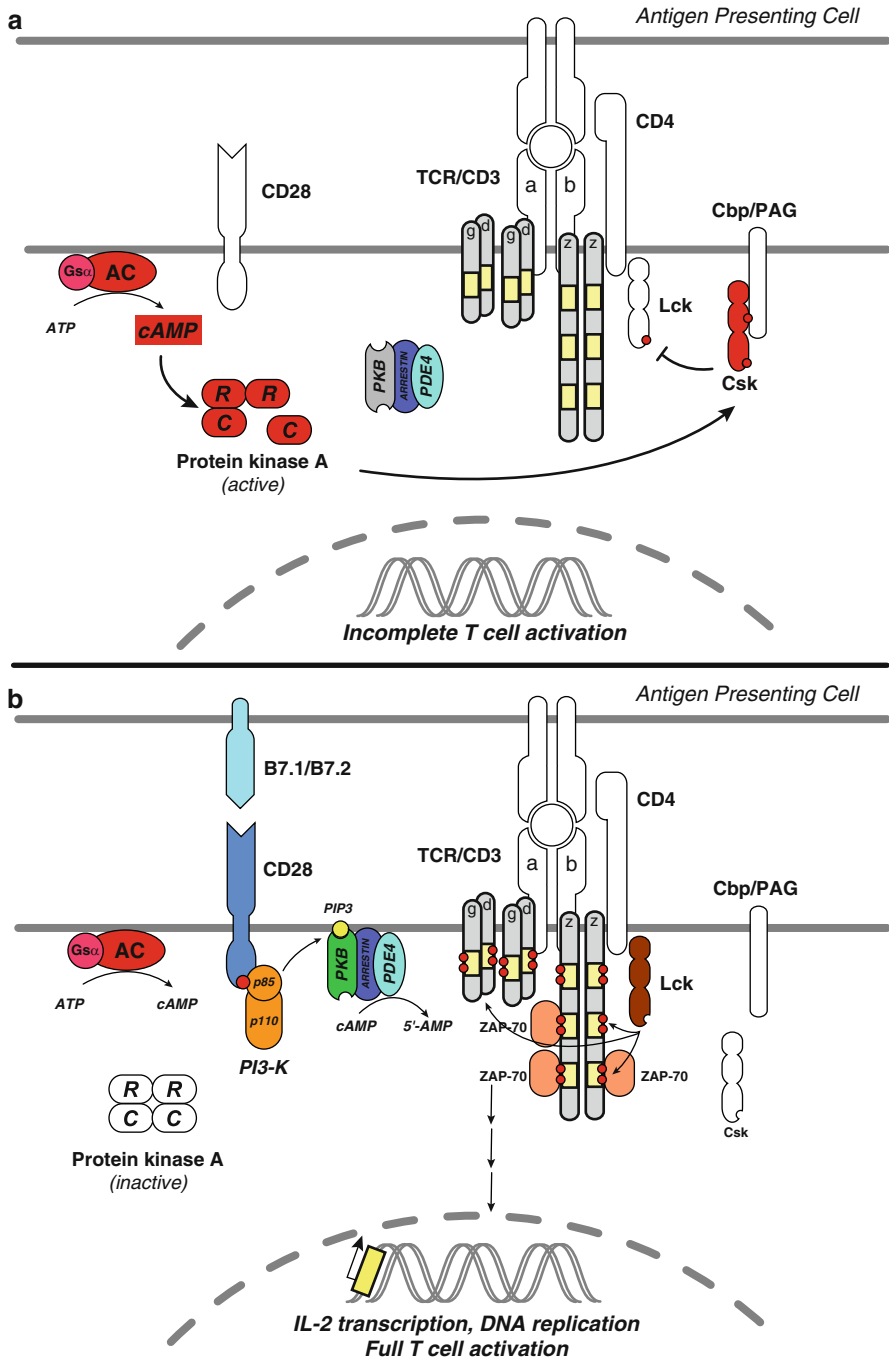


Fig. 2 PKA and PDE4 have opposing functions in proximal T-cell signaling. TCR ligation results in rapid cAMP production in lipid rafts due to G protein coupling to raft-associated adenylyl cyclase.

Perry et al. 2002). In addition to interacting with a common site in the catalytic domain of all PDE4Ds (Perry et al. 2002), β -arrestins also interact specifically with the N-terminus of PDE4D5, making this isoform a preferred binding partner for β -arrestin (Bolger et al. 2003). Intriguingly, we found that TCR/CD28 costimulation led to profound recruitment of β -arrestin to T-cell lipid raft fractions concurrently with PDE4 and that β -arrestin and PDE4D preexist in a complex prior to stimulation, indicating that they are recruited to rafts together (Abrahamsen et al. 2004).

TCR-induced cAMP production in lipid rafts results in PKA activation and the subsequent inhibition of proximal T-cell signaling. However, overexpression of PDE4 isoforms or β -arrestin has been demonstrated to increase T-cell activation, revealing regulatory roles for both proteins in T-cell signaling (Abrahamsen et al. 2004). In conclusion, the activities of both PKA and PDE4, therefore, seem to be important for the regulation of TCR-induced signaling and T-cell function. We propose a novel role for TCR and CD28 costimulation in downmodulation of TCR-induced cAMP-mediated inhibitory signals through the recruitment of a PDE4/ β -arrestin complex to lipid rafts, thus allowing a full T-cell response to occur. Interestingly, the cAMP inhibitory pathway has been implicated in several immune diseases. HIV-infected patients have T cells with elevated cAMP levels and hyperactivated PKA, and targeting the cAMP–PKA–Csk inhibitory pathway by selective antagonists reverses T-cell dysfunction in HIV cells *ex vivo* (Aandahl et al. 1998, 1999). Improved *in vivo* T-cell function has been observed targeting COX-2, thereby reducing PGE₂ production and reducing the cAMP levels (Johansson et al. 2004; Kvale et al. 2006). Furthermore, a high proportion of patients with common variable immunodeficiency (CVID) show T-cell abnormalities. Recent findings demonstrating increased PKA activation and impaired IL-10 secretion by T cells from these patients suggest a link between T-cell deficiency and impaired B-cell function in CVID, indicating that the cAMP–PKA pathway is a target for therapeutic immunomodulation in CVID (Aukrust et al. 1999; Holm et al. 2003).

1.3.2 PDE4/ β -Arrestin Recruitment to T-Cell Lipid Rafts is Regulated by Phosphatidylinositol 3-Kinase (PI3K)

Recruitment of β -arrestin and PDE4 to lipid rafts was not only observed upon TCR/CD28 costimulation. CD28 stimulation alone seems to be enough to recruit these proteins, whereas only very low levels comparable to basal levels were observed

←

Fig. 2 (continued) Increased cAMP levels lead to activation of PKA, phosphorylation and activation of Csk, and inhibition of Lck, thus preventing full T-cell activation (**a**). However, TCR/CD28 costimulation downmodulates the TCR-induced cAMP-mediated inhibitory signals through recruitment of a PKB/ β -arrestin/PDE4 complex leading to cAMP degradation, thus allowing a full T-cell response to occur. Recruitment of this complex is dependent on phosphatidylinositol 3-kinase (PI3K) activity and PIP₃ production, and the complex is targeted to the membrane via the pleckstrin homology (PH) domain of PKB (**b**)

upon TCR stimulation (Abrahamsen et al. 2004; Bjørge et al. 2010). Furthermore, CD28 ligation was shown to act synergistically with CD3 stimulation in the recruitment of β -arrestin and PDE4, and we concluded that the CD28 signal plays a distinct role enhanced by a concomitant TCR signal (Bjørge et al. 2010).

The CD28 coreceptor plays a central role in T-cell activation *in vivo*. Although engagement of the TCR provides the necessary scaffolding from which TCR signaling is propagated, CD28 functions as an amplifier necessary to promote optimal IL-2 production and clonal expansion and to prevent anergy and cell death. The CD28-mediated signals are transmitted via a short intracellular stretch in the receptor containing conserved motifs important for docking of several signaling proteins (Michel et al. 2001). Phosphorylation of a tyrosine residue (Y173) in the conserved YNMN motif by Lck and Fyn is a key to efficient signal transduction (Raab et al. 1995). This generates a docking site for the SH2 domain of p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (Pages et al. 1994; Prasad et al. 1994). The C-terminal PXXP motif is another important binding site recognized by both Src family kinases and Grb2 (Holdorf et al. 1999; Kim et al. 1998; Okkenhaug and Rottapel 1998). It has been somewhat controversial whether CD28 alone can induce PI3K activity. However, phosphorylation of the PKB substrate glycogen synthase kinase 3 (GSK3) upon CD28 stimulation has recently been observed (Appleman et al. 2002; Diehn et al. 2002), demonstrating that PI3K is indeed activated upon CD28 stimulation alone. Thus, we examined how β -arrestin and PDE4 levels in T-cell lipid rafts were affected by the inhibition of either SFKs or PI3K using PP2 and LY294002, respectively. Strikingly, CD28-induced recruitment of β -arrestin in primary T cells was abolished upon both Lck/Fyn and PI3K inhibition. Furthermore, PDE4 activity in raft fractions from T cells pretreated with the PI3K inhibitor prior to CD28 stimulation was reduced below basal levels and no increase in PDE4 activity was observed upon CD28 ligation (Bjørge et al. 2010). Taken together, these data suggest a novel mechanism, whereby PI3K can regulate cAMP degradation through recruitment of a PDE4/ β -arrestin complex to lipid rafts.

1.3.3 The PDE4/ β -Arrestin Complex Interacts with PKB

Activation of PI3K results in rapid phosphatidylinositol 3,4,5-trisphosphate (PIP3) generation in the inner leaflet of the plasma membrane and recruitment of proteins containing a specialized lipid-binding moiety called the pleckstrin homology (PH) domain. Thus, PIP3 controls both activity and subcellular localization of a broad range of signal transduction molecules (Kane and Weiss 2003). Vav-1, Itk, and PKB are three PH-containing proteins that have been implicated both downstream of PI3K signaling and in TCR/CD28 costimulation (Acuto et al. 2003). We recently demonstrated that β -arrestin interacts with PIP3 in an activation-dependent fashion, although β -arrestin does not possess a PH domain (Bjørge et al. 2010). We, therefore, hypothesized the presence of a PH domain-containing interaction partner inside the complex that confers binding to PIP3 and can mediate the translocation of

the PDE4D/ β -arrestin complex upon TCR/CD28 costimulation (Bjorgo et al. 2010). Interestingly, PKB was shown to be recruited to lipid rafts concurrently with β -arrestin in TCR/CD28 costimulated T cells. In addition, we found that PKB coimmunoprecipitated with β -arrestin in TCR/CD28 costimulated T cells, and it was further demonstrated that PKB, β -arrestin, and PDE4 through defined contact areas indeed form a trimolecular interaction where β -arrestin interacts directly with both PKB and PDE4 (Bjorgo et al. 2010).

Binding of the PKB PH domain to PIP3 recruits PKB from the cytosol and is also thought to induce a conformational change that converts PKB into a substrate that can be activated by the associated phosphoinositide-dependent kinase 1 (PDK1) (Milburn et al. 2003). Lately, several reports have demonstrated that β -arrestin can function as a signaling scaffold by assembling either positive or negative regulators of the PKB pathway. More specifically, β -arrestin 2 has been reported to be involved in PKB signaling downstream of the dopamine receptor in neurons where dopamine stimulates formation of a signaling complex consisting of β -arrestin 2, PKB, and protein phosphatase 2A (PP2A) (Beaulieu et al. 2005). Furthermore, the PI3K–PKB pathway has been reported to be activated in a β -arrestin 1-dependent manner upon stimulation of the insulin-like growth factor (IGF-1) receptor, resulting in increased protection from apoptosis (Povsic et al. 2003). It has also been demonstrated that upon insulin stimulation, β -arrestin 2 mediates PKB activation through Src family tyrosine kinase in a PI3K-independent way (Luan et al. 2009). We have recently demonstrated that PKB in T cells, through its PH domain, is responsible for transporting the PDE4D/ β -arrestin/PKB complex to the membrane upon TCR/CD28 costimulation, thus revealing a novel role for PKB (Bjorgo et al. 2010).

1.3.4 Functional Importance of the PKB/ β -Arrestin/PDE4 Complex

Although the exact role of β -arrestin in proximal T-cell signaling is still not fully understood, siRNA-mediated knockdown of β -arrestins 1 and 2 in human primary T cells resulted in a 40 and 30% reduction in IL-2 and IFN- γ production, respectively, indicating that β -arrestin plays a positive regulatory role in proximal T-cell activation (Bjorgo et al. 2010). The observed reduction in IL-2 production is comparable to the effect observed by pharmacologically inhibiting PDE4 activity with rolipram, suggesting that the main function of β -arrestin in proximal T-cell signaling resides in its ability to recruit the signal termination enzyme PDE4 to lipid rafts and thereby break the cAMP negative feedback governing T-cell receptor functioning. Taken together, PKA and PDE4 isoforms seem to have opposing functions during proximal T-cell signaling, thereby titrating the activation-induced response. Thus, a novel mechanism of signaling by CD28 is revealed where CD28 through PI3K regulates cAMP degradation in lipid rafts via recruitment of a PDE4/ β -arrestin/PKB complex, thereby allowing a complete T-cell activation to proceed.

T-cell proliferation and production of the cytokines IL-2, IL-4, IL-5, IFN- γ , and TNF- α are blocked by PDE4 inhibitors (Jimenez 2001). These anti-inflammatory

properties of PDE4 inhibition can be of significant clinical benefit, and PDE4 inhibitors are currently being developed to treat COPD, asthma, and other inflammatory conditions (Houslay et al. 2005). Recently, two large phase III clinical trials using the PDE4-specific inhibitor roflumilast (Daxas) as treatment of COPD were reported (Calverley et al. 2009; Fabbri et al. 2009), showing significant improvement in lung function. However, side effects such as nausea, diarrhea, weight loss, and headache were also reported. Roflumilast was approved in the European Commission in July 2010 for treatment of severe COPD associated with chronic bronchitis in adult patients but has still not obtained FDA approval.

1.4 The Functional Role of PDE7 in T Cells

The PDE7 gene family comprises two genes, PDE7A and PDE7B, and PDE7A transcripts have been shown to be widely expressed in immune cells (Bloom and Beavo 1996; Gardner et al. 2000; Hetman et al. 2000). More specifically, expression of PDE7A1 is restricted mainly to T cells and brain, and PDE7A3 is expressed in activated CD4⁺ T cells (Bloom and Beavo 1996; Glavas et al. 2001; Smith et al. 2003). Whereas resting T cells mainly express PDE3 and PDE4, TCR/CD28 costimulated cells upregulate their PDE7 levels (Li et al. 1999). The functional role of PDE7 in T cells has, however, been controversial. PDE7A1 was reported to be required to obtain proper IL-2 production and proliferation in TCR/CD28 costimulated T cells in a study using targeted antisense oligonucleotides (Li et al. 1999). However, T cells from PDE7A-deficient mice did not reveal any deficiencies in T-cell function (Yang et al. 2003). The expression profiles of PDE7A transcripts indicate that PDE7-selective inhibitors could have a broad application as immunosuppressants, and recently, PDE7 inhibitors have become available (Guo et al. 2009; Kadoshima-Yamaoka et al. 2009). One PDE7A inhibitor increased the effect of rolipram in T cells without having a similar effect itself (Smith et al. 2004). A recent study used the PDE7 inhibitor ASB16165 to examine the functional role of PDE7 in generation and function of cytotoxic T lymphocytes (CTL), and it was suggested that PDE7 and not PDE4 plays the major role in induction and function of CTL in mice (Kadoshima-Yamaoka et al. 2009). Development of dual PDE4–PDE7 inhibitors is another strategy that could result in drugs blocking T-cell components of a disease by inhibiting PDE7 in addition to possessing anti-inflammatory properties (Giembycz 2005; Yamamoto et al. 2007). However, more work is required before the exact role of PDE7 in controlling immune responses can be teased out and PDE7 inhibitors positioned therapeutically.

2 Concluding Remarks

PDEs play a key role in compartmentalization of cAMP signaling. Scaffolding and spatiotemporal control of receptors, cyclases, and PKA by AKAPs in addition to generation of local pools of cAMP within the cell by the action of PDEs give rise to

a high degree of specificity in PKA-mediated signaling despite the multiplicity of receptors and targets using cAMP as a second messenger. Additional specificity is obtained by integrating PDEs into these anchoring complexes and by the presence of a large number of PDE isoforms distributed in different tissues and at varying levels. The use of PDE-specific inhibitors has revealed that especially PDE4 isoforms are important drug targets in several diseases such as asthma, chronic obstructive pulmonary disease (COPD), cardiovascular diseases, and neurological disorders. In addition, PDEs are also interesting drug targets for immunosuppression since cAMP potently inhibits immune functions.

References

- Aandahl EM, Aukrust P, Skalhegg BS, Muller F, Froland SS, Hansson V, Tasken K (1998) Protein kinase A type I antagonist restores immune responses of T cells from HIV-infected patients. *FASEB J* 12:855–862
- Aandahl EM, Aukrust P, Muller F, Hansson V, Tasken K, Froland SS (1999) Additive effects of IL-2 and protein kinase A type I antagonist on function of T cells from HIV-infected patients on HAART. *AIDS* 13:F109–F114
- Aandahl EM, Moretto WJ, Haslett PA, Vang T, Bryn T, Tasken K, Nixon DF (2002) Inhibition of antigen-specific T cell proliferation and cytokine production by protein kinase A type I. *J Immunol* 169:802–808
- Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF (2004) Human CD4⁺ CD25⁺ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 78:2454–2459
- Abrahamsen H, Baillie G, Ngai J, Vang T, Nika K, Ruppelt A, Mustelin T, Zaccolo M, Houslay MD, Tasken K (2004) TCR- and CD28-mediated recruitment of phosphodiesterase 4 to lipid rafts potentiates T cell receptor signaling. *J Immunol* 173:4847–4858
- Acuto O, Mise-Omata S, Mangino G, Michel F (2003) Molecular modifiers of T cell antigen receptor triggering threshold: the mechanism of CD28 costimulatory receptor. *Immunol Rev* 192:21–31
- Appleman LJ, van Puijenbroek AA, Shu KM, Nadler LM, Boussiotis VA (2002) CD28 costimulation mediates down-regulation of p27kip1 and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells. *J Immunol* 168:2729–2736
- Arp J, Kirchhof MG, Baroja ML, Nazarian SH, Chau TA, Strathdee CA, Ball EH, Madrenas J (2003) Regulation of T-cell activation by phosphodiesterase 4B2 requires its dynamic redistribution during immunological synapse formation. *Mol Cell Biol* 23:8042–8057
- Aukrust P, Aandahl EM, Skalhegg BS, Nordoy I, Hansson V, Tasken K, Froland SS, Muller F (1999) Increased activation of protein kinase A type I contributes to the T cell deficiency in common variable immunodeficiency. *J Immunol* 162:1178–1185
- Baillie GS, Sood A, McPhee I, Gall I, Perry SJ, Lefkowitz RJ, Houslay MD (2003) beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from Gs to Gi. *Proc Natl Acad Sci USA* 100:940–945
- Baillie GS, Scott JD, Houslay MD (2005) Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. *FEBS Lett* 579:3264–3270
- Baroja ML, Cieslinski LB, Torphy TJ, Wange RL, Madrenas J (1999) Specific CD3 epsilon association of a phosphodiesterase 4B isoform determines its selective tyrosine phosphorylation after CD3 ligation. *J Immunol* 162:2016–2023
- Bazhin AV, Kahnert S, Kimpfler S, Schadendorf D, Umansky V (2010) Distinct metabolism of cyclic adenosine monophosphate in regulatory and helper CD4⁺ T cells. *Mol Immunol* 47:678–684

- Beaulieu JM, Sotnikova TD, Marion S, Lefkowitz RJ, Gainetdinov RR, Caron MG (2005) An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* 122:261–273
- Bergman M, Mustelin T, Oetken C, Partanen J, Flint NA, Amrein KE, Autero M, Burn P, Alitalo K (1992) The human p50csk tyrosine kinase phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity. *EMBO J* 11:2919–2924
- Beyer M, Schultze JL (2006) Regulatory T cells in cancer. *Blood* 108:804–811
- Bjorgo E, Solheim SA, Abrahamsen H, Baillie GS, Brown KM, Berge T, Okkenhaug K, Houslay MD, Tasken K (2010) Crosstalk between PI3-Kinase and cAMP-protein kinase A signaling pathways at the level of a Protein Kinase B/{beta}-arrestin/cAMP phosphodiesterase-4 complex. *Mol Cell Biol* 30:1660–1672
- Bloom TJ, Beavo JA (1996) Identification and tissue-specific expression of PDE7 phosphodiesterase splice variants. *Proc Natl Acad Sci USA* 93:14188–14192
- Bluestone JA, Abbas AK (2003) Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 3:253–257
- Bolger GB, McCahill A, Huston E, Cheung YF, McSorley T, Baillie GS, Houslay MD (2003) The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with beta-arrestins. *J Biol Chem* 278:49230–49238
- Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Heib V, Becker M, Kubach J, Schmitt S, Stoll S, Schild H, Staeger MS, Stassen M, Jonuleit H, Schmitt E (2007) Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med* 204:1303–1310
- Brdicka T, Pavlistova D, Leo A, Bruyns E, Korinek V, Angelisova P, Scherer J, Shevchenko A, Hilgert I, Cerny J, Drbal K, Kuramitsu Y, Kornacker B, Horejsi V, Schraven B (2000) Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. *J Exp Med* 191:1591–1604
- Calverley PM, Rabe KF, Goehring UM, Kristiansen S, Fabbri LM, Martinez FJ (2009) Roflumilast in symptomatic chronic obstructive pulmonary disease: two randomised clinical trials. *Lancet* 374:685–694
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- Conti M, Jin SL (1999) The molecular biology of cyclic nucleotide phosphodiesterases. *Prog Nucleic Acid Res Mol Biol* 63:1–38
- Corbin JD, Francis SH, Webb DJ (2002) Phosphodiesterase type 5 as a pharmacologic target in erectile dysfunction. *Urology* 60:4–11
- Davidson D, Bakinowski M, Thomas ML, Horejsi V, Veillette A (2003) Phosphorylation-dependent regulation of T-cell activation by PAG/Cbp, a lipid raft-associated transmembrane adaptor. *Mol Cell Biol* 23:2017–2028
- de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 396:474–477
- Deaglio S, Dwyer KM, Gao W, Friedman D, Usheva A, Erat A, Chen JF, Enjoji K, Linden J, Oukka M, Kuchroo VK, Strom TB, Robson SC (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204:1257–1265
- DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK (2007) Beta-arrestins and cell signaling. *Annu Rev Physiol* 69:483–510
- Diehn M, Alizadeh AA, Rando OJ, Liu CL, Stankunas K, Botstein D, Crabtree GR, Brown PO (2002) Genomic expression programs and the integration of the CD28 costimulatory signal in T cell activation. *Proc Natl Acad Sci USA* 99:11796–11801
- Erdogan S, Houslay MD (1997) Challenge of human Jurkat T-cells with the adenylate cyclase activator forskolin elicits major changes in cAMP phosphodiesterase (PDE) expression by

- up-regulating PDE3 and inducing PDE4D1 and PDE4D2 splice variants as well as down-regulating a novel PDE4A splice variant. *Biochem J* 321(Pt 1):165–175
- Essayan DM (2001) Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol* 108:671–680
- Fabbri LM, Calverley PM, Izquierdo-Alonso JL, Bundschuh DS, Brose M, Martinez FJ, Rabe KF (2009) Roflumilast in moderate-to-severe chronic obstructive pulmonary disease treated with longacting bronchodilators: two randomised clinical trials. *Lancet* 374:695–703
- Feldman AM, McNamara DM (2002) Reevaluating the role of phosphodiesterase inhibitors in the treatment of cardiovascular disease. *Clin Cardiol* 25:256–262
- Francis SH, Corbin JD (2005) Phosphodiesterase-5 inhibition: the molecular biology of erectile function and dysfunction. *Urol Clin North Am* 32:419–429, vi
- Gardner C, Robas N, Cawkill D, Fidock M (2000) Cloning and characterization of the human and mouse PDE7B, a novel cAMP-specific cyclic nucleotide phosphodiesterase. *Biochem Biophys Res Commun* 272:186–192
- Gavin MA, Rasmussen JP, Fontenot JD, Vasta V, Manganiello VC, Beavo JA, Rudensky AY (2007) Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 445:771–775
- Giembycz MA (2005) Phosphodiesterase-4: selective and dual-specificity inhibitors for the therapy of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2:326–333
- Giembycz MA, Corrigan CJ, Seybold J, Newton R, Barnes PJ (1996) Identification of cyclic AMP phosphodiesterases 3, 4 and 7 in human CD4⁺ and CD8⁺ T-lymphocytes: role in regulating proliferation and the biosynthesis of interleukin-2. *Br J Pharmacol* 118:1945–1958
- Glavas NA, Ostenson C, Schaefer JB, Vasta V, Beavo JA (2001) T cell activation up-regulates cyclic nucleotide phosphodiesterases 8A1 and 7A3. *Proc Natl Acad Sci USA* 98:6319–6324
- Gretarsdottir S, Thorleifsson G, Reynisdottir ST, Manolescu A, Jonsdottir S, Jonsdottir T, Gudmundsdottir T, Bjarnadottir SM, Einarsson OB, Gudjonsdottir HM, Hawkins M, Gudmundsson G, Gudmundsdottir H, Andrason H, Gudmundsdottir AS, Sigurdardottir M, Chou TT, Nahmias J, Goss S, Sveinbjornsdottir S, Valdimarsson EM, Jakobsson F, Agnarsson U, Gudnason V, Thorgeirsson G, Fingerle J, Gurney M, Gudbjartsson D, Frigge ML, Kong A, Stefansson K, Gulcher JR (2003) The gene encoding phosphodiesterase 4D confers risk of ischemic stroke. *Nat Genet* 35:131–138
- Guo J, Watson A, Kempson J, Carlsen M, Barbosa J, Stebbins K, Lee D, Dodd J, Nadler SG, McKinnon M, Barrish J, Pitts WJ (2009) Identification of potent pyrimidine inhibitors of phosphodiesterase 7 (PDE7) and their ability to inhibit T cell proliferation. *Bioorg Med Chem Lett* 19:1935–1938
- Hanoue J, Defer N (2001) Regulation and role of adenylyl cyclase isoforms. *Annu Rev Pharmacol Toxicol* 41:145–174
- Hansen G, Jin S, Umetsu DT, Conti M (2000) Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. *Proc Natl Acad Sci USA* 97:6751–6756
- Hetman JM, Robas N, Baxendale R, Fidock M, Phillips SC, Soderling SH, Beavo JA (2000) Cloning and characterization of two splice variants of human phosphodiesterase 11A. *Proc Natl Acad Sci USA* 97:12891–12895
- Holdorf AD, Green JM, Levin SD, Denny MF, Straus DB, Link V, Changelian PS, Allen PM, Shaw AS (1999) Proline residues in CD28 and the Src homology (SH)3 domain of Lck are required for T cell costimulation. *J Exp Med* 190:375–384
- Holm AM, Aukrust P, Aandahl EM, Muller F, Tasken K, Froland SS (2003) Impaired secretion of IL-10 by T cells from patients with common variable immunodeficiency—involvement of protein kinase A type I. *J Immunol* 170:5772–5777
- Houslay MD (2009) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 35:91–100
- Houslay MD, Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J* 370:1–18
- Houslay MD, Schafer P, Zhang KY (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10:1503–1519

- Houslay MD, Baillie GS, Maurice DH (2007) cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. *Circ Res* 100:950–966
- Jimenez JL, Punzón C, Navarro J, Muñoz-Fernandez MA, Fresno M (2001) Phosphodiesterase 4 inhibitors prevent cytokine secretion by T lymphocytes by inhibiting nuclear factor-kappa B and nuclear factor of activated T cells activation. *J pharmacol Exp Ther* 299:753–759
- Jin SL, Conti M (2002) Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-alpha responses. *Proc Natl Acad Sci USA* 99:7628–7633
- Jin SL, Richard FJ, Kuo WP, D'Ercole AJ, Conti M (1999) Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice. *Proc Natl Acad Sci USA* 96:11998–12003
- Jin SL, Lan L, Zoudilova M, Conti M (2005) Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. *J Immunol* 175:1523–1531
- Johansson CC, Bryn T, Yndestad A, Eiken HG, Bjerkeli V, Froland SS, Aukrust P, Tasken K (2004) Cytokine networks are pre-activated in T cells from HIV-infected patients on HAART and are under the control of cAMP. *AIDS* 18:171–179
- Jordan MS, Singer AL, Koretzky GA (2003) Adaptors as central mediators of signal transduction in immune cells. *Nat Immunol* 4:110–116
- Kadoshima-Yamaoka K, Murakawa M, Goto M, Tanaka Y, Inoue H, Murafuji H, Hayashi Y, Nagahira K, Miura K, Nakatsuka T, Chamoto K, Fukuda Y, Nishimura T (2009) Effect of phosphodiesterase 7 inhibitor ASB16165 on development and function of cytotoxic T lymphocyte. *Int Immunopharmacol* 9:97–102
- Kane LP, Weiss A (2003) The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. *Immunol Rev* 192:7–20
- Kaupp UB, Seifert R (2002) Cyclic nucleotide-gated ion channels. *Physiol Rev* 82:769–824
- Kawabuchi M, Satomi Y, Takao T, Shimonishi Y, Nada S, Nagai K, Tarakhovskiy A, Okada M (2000) Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. *Nature* 404:999–1003
- Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM (1998) A family of cAMP-binding proteins that directly activate Rap1. *Science* 282:2275–2279
- Ke H, Wang H (2007) Crystal structures of phosphodiesterases and implications on substrate specificity and inhibitor selectivity. *Curr Top Med Chem* 7:391–403
- Kim HH, Tharayil M, Rudd CE (1998) Growth factor receptor-bound protein 2 SH2/SH3 domain binding to CD28 and its role in co-signaling. *J Biol Chem* 273:296–301
- Kvale D, Ormaasen V, Kran AM, Johansson CC, Aukrust P, Aandahl EM, Froland SS, Tasken K (2006) Immune modulatory effects of cyclooxygenase type 2 inhibitors in HIV patients on combination antiretroviral treatment. *AIDS* 20:813–820
- Ledbetter JA, Parsons M, Martin PJ, Hansen JA, Rabinovitch PS, June CH (1986) Antibody binding to CD5 (Tp67) and Tp44 T cell surface molecules: effects on cyclic nucleotides, cytoplasmic free calcium, and cAMP-mediated suppression. *J Immunol* 137:3299–3305
- Li L, Yee C, Beavo JA (1999) CD3- and CD28-dependent induction of PDE7 required for T cell activation. *Science* 283:848–851
- Luan B, Zhao J, Wu H, Duan B, Shu G, Wang X, Li D, Jia W, Kang J, Pei G (2009) Deficiency of a beta-arrestin-2 signal complex contributes to insulin resistance. *Nature* 457:1146–1149
- Lynch MJ, Baillie GS, Mohamed A, Li X, Maisonneuve C, Klussmann E, van Heeke G, Houslay MD (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated switching of the beta2-adrenergic receptor to activation of ERK in HEK293B2 cells. *J Biol Chem* 280:33178–33189
- Mahic M, Yaqub S, Johansson CC, Tasken K, Aandahl EM (2006) FOXP3⁺CD4⁺CD25⁺ adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E2-dependent mechanism. *J Immunol* 177:246–254

- Manji HK, Quiroz JA, Sporn J, Payne JL, Denicoff K, Gray A, Zarate CA Jr, Charney DS (2003) Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. *Biol Psychiatry* 53:707–742
- Masciarelli S, Horner K, Liu C, Park SH, Hinckley M, Hockman S, Nedachi T, Jin C, Conti M, Manganiello V (2004) Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. *J Clin Invest* 114:196–205
- Matulef K, Zagotta WN (2003) Cyclic nucleotide-gated ion channels. *Annu Rev Cell Dev Biol* 19:23–44
- McCahill A, McSorley T, Huston E, Hill EV, Lynch MJ, Gall I, Keryer G, Lygren B, Tasken K, van Heeke G, Houslay MD (2005) In resting COS1 cells a dominant negative approach shows that specific, anchored PDE4 cAMP phosphodiesterase isoforms gate the activation, by basal cyclic AMP production, of AKAP-tethered protein kinase A type II located in the centrosomal region. *Cell Signal* 17:1158–1173
- Mehats C, Andersen CB, Filopanti M, Jin SL, Conti M (2002) Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. *Trends Endocrinol Metab* 13:29–35
- Michel JJ, Scott JD (2002) AKAP mediated signal transduction. *Annu Rev Pharmacol Toxicol* 42:235–257
- Michel F, Attal-Bonnefoy G, Mangino G, Mise-Omata S, Acuto O (2001) CD28 as a molecular amplifier extending TCR ligation and signaling capabilities. *Immunity* 15:935–945
- Milburn CC, Deak M, Kelly SM, Price NC, Alessi DR, Van Aalten DM (2003) Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. *Biochem J* 375:531–538
- Millar JK, Pickard BS, Mackie S, James R, Christie S, Buchanan SR, Malloy MP, Chubb JE, Huston E, Baillie GS, Thomson PA, Hill EV, Brandon NJ, Rain JC, Camargo LM, Whiting PJ, Houslay MD, Blackwood DH, Muir WJ, Porteous DJ (2005) DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. *Science* 310:1187–1191
- Montixi C, Langlet C, Bernard AM, Thimonier J, Dubois C, Wurbel MA, Chauvin JP, Pierres M, He HT (1998) Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J* 17:5334–5348
- Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD, Isaacs JD, Lechler RI (2001) Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 98:2736–2744
- Oh P, Schnitzer JE (2001) Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default. *Mol Biol Cell* 12:685–698
- Okada M, Nakagawa H (1989) A protein tyrosine kinase involved in regulation of pp 60c-src function. *J Biol Chem* 264:20886–20893
- Okkenhaug K, Rottapel R (1998) Grb2 forms an inducible protein complex with CD28 through a Src homology 3 domain-proline interaction. *J Biol Chem* 273:21194–21202
- Pages F, Ragueneau M, Rottapel R, Truneh A, Nunes J, Imbert J, Olive D (1994) Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature* 369:327–329
- Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, Lefkowitz RJ (2002) Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. *Science* 298:834–836
- Povsic TJ, Kohout TA, Lefkowitz RJ (2003) Beta-arrestin1 mediates insulin-like growth factor 1 (IGF-1) activation of phosphatidylinositol 3-kinase (PI3K) and anti-apoptosis. *J Biol Chem* 278:51334–51339
- Prasad KV, Cai YC, Raab M, Duckworth B, Cantley L, Shoelson SE, Rudd CE (1994) T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc Natl Acad Sci USA* 91:2834–2838
- Prins GS, Tang WY, Belmonte J, Ho SM (2008) Perinatal exposure to oestradiol and bisphenol A alters the prostate epigenome and increases susceptibility to carcinogenesis. *Basic Clin Pharmacol Toxicol* 102:134–138

- Raaf M, Cai YC, Bunnell SC, Heyeck SD, Berg LJ, Rudd CE (1995) p56Lck and p59Fyn regulate CD28 binding to phosphatidylinositol 3-kinase, growth factor receptor-bound protein GRB-2, and T cell-specific protein-tyrosine kinase ITK: implications for T-cell costimulation. *Proc Natl Acad Sci USA* 92:8891–8895
- Rouse BT, Sarangi PP, Suvas S (2006) Regulatory T cells in virus infections. *Immunol Rev* 212:272–286
- Rudd CE (1999) Adaptors and molecular scaffolds in immune cell signaling. *Cell* 96:5–8
- Ruppelt A, Mosenden R, Gronholm M, Aandahl EM, Tobin D, Carlson CR, Abrahamsen H, Herberg FW, Carpen O, Tasken K (2007) Inhibition of T cell activation by cyclic adenosine 5'-monophosphate requires lipid raft targeting of protein kinase A type I by the A-kinase anchoring protein ezrin. *J Immunol* 179:5159–5168
- Sakaguchi S (2004) Naturally arising CD4⁺ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22:531–562
- Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M, Takahashi T (2001) Immunologic tolerance maintained by CD25⁺ CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 182:18–32
- Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T (2006) Foxp3⁺ CD25⁺ CD4⁺ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212:8–27
- Skalhegg BS, Landmark BF, Doskeland SO, Hansson V, Lea T, Jahnsen T (1992) Cyclic AMP-dependent protein kinase type I mediates the inhibitory effects of 3', 5'-cyclic adenosine monophosphate on cell replication in human T lymphocytes. *J Biol Chem* 267:15707–15714
- Smith FD, Scott JD (2002) Signaling complexes: junctions on the intracellular information super highway. *Curr Biol* 12:R32–R40
- Smith SJ, Brookes-Fazakerley S, Donnelly LE, Barnes PJ, Barnette MS, Giembycz MA (2003) Ubiquitous expression of phosphodiesterase 7A in human proinflammatory and immune cells. *Am J Physiol Lung Cell Mol Physiol* 284:L279–L289
- Smith SJ, Cieslinski LB, Newton R, Donnelly LE, Fenwick PS, Nicholson AG, Barnes PJ, Barnette MS, Giembycz MA (2004) Discovery of BRL 50481 [3-(N, N-dimethylsulfonamido)-4-methyl-nitrobenzene], a selective inhibitor of phosphodiesterase 7: in vitro studies in human monocytes, lung macrophages, and CD8⁺ T-lymphocytes. *Mol Pharmacol* 66:1679–1689
- Spina D (2003) Theophylline and PDE4 inhibitors in asthma. *Curr Opin Pulm Med* 9:57–64
- Spina D (2008) PDE4 inhibitors: current status. *Br J Pharmacol* 155:308–315
- Tasken K, Aandahl EM (2004) Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol Rev* 84:137–167
- Thompson PE, Manganiello V, Degerman E (2007) Re-discovering PDE3 inhibitors—new opportunities for a long neglected target. *Curr Top Med Chem* 7:421–436
- Torgersen KM, Vang T, Abrahamsen H, Yaqub S, Horejsi V, Schraven B, Rolstad B, Mustelin T, Tasken K (2001) Release from tonic inhibition of T cell activation through transient displacement of C-terminal Src kinase (Csk) from lipid rafts. *J Biol Chem* 276:29313–29318
- Torgersen KM, Vang T, Abrahamsen H, Yaqub S, Tasken K (2002) Molecular mechanisms for protein kinase A-mediated modulation of immune function. *Cell Signal* 14:1–9
- Vang T, Torgersen KM, Sundvold V, Saxena M, Levy FO, Skalhegg BS, Hansson V, Mustelin T, Tasken K (2001) Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. *J Exp Med* 193:497–507
- Vang T, Abrahamsen H, Myklebust S, Horejsi V, Tasken K (2003) Combined spatial and enzymatic regulation of Csk by cAMP and protein kinase a inhibits T cell receptor signaling. *J Biol Chem* 278:17597–17600
- Vlachopoulos C, Terentes-Prinzios D, Ioakeimidis N, Rokkas K, Stefanadis C (2009) PDE5 inhibitors in non-urolological conditions. *Curr Pharm Des* 15:3521–3539

- Walsh DA, Perkins JP, Krebs EG (1968) An adenosine 3', 5'-monophosphate-dependant protein kinase from rabbit skeletal muscle. *J Biol Chem* 243:3763–3765
- Xavier R, Brennan T, Li Q, McCormack C, Seed B (1998) Membrane compartmentation is required for efficient T cell activation. *Immunity* 8:723–732
- Yamaguchi H, Hendrickson WA (1996) Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. *Nature* 384:484–489
- Yamamoto S, Sugahara S, Ikeda K, Shimizu Y (2007) Amelioration of collagen-induced arthritis in mice by a novel phosphodiesterase 7 and 4 dual inhibitor, YM-393059. *Eur J Pharmacol* 559:219–226
- Yang G, McIntyre KW, Townsend RM, Shen HH, Pitts WJ, Dodd JH, Nadler SG, McKinnon M, Watson AJ (2003) Phosphodiesterase 7A-deficient mice have functional T cells. *J Immunol* 171:6414–6420
- Yaqub S, Tasken K (2008) Role for the cAMP-protein kinase A signaling pathway in suppression of antitumor immune responses by regulatory T cells. *Crit Rev Oncog* 14:57–77
- Zaccolo M, Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science* 295:1711–1715
- Zhang W, Sloan-Lancaster J, Kitchen J, Tribble RP, Samelson LE (1998) LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92:83–92

Cyclic Nucleotides and Phosphodiesterases in Monocytic Differentiation

Angie L. Hertz and Joseph A. Beavo

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Abstract Monocytes are immune cells that can differentiate into a number of cell types including macrophages, dendritic cells, and osteoclasts upon exposure to various cytokines. The phenotypes of these differentiated cells are highly heterogeneous and their differentiation can be affected by the cyclic nucleotides, 3'-5'-cyclic adenosine monophosphate (cAMP) and 3'-5'-cyclic guanosine monophosphate (cGMP). The intracellular levels of cAMP and cGMP are controlled through regulation of production by adenylyl and guanylyl cyclases and through degradation by cyclic nucleotide phosphodiesterases (PDEs). PDE inhibition and subsequent changes in cyclic nucleotide levels can alter the final phenotype of a differentiating monocyte with regards to surface marker expression, gene expression, or changes in secreted chemokine and cytokine levels. The differentiation process itself can also be either inhibited or augmented by changes in cyclic nucleotide levels, depending on the system being studied and the timing of cyclic nucleotide elevation. This chapter

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explores the effects of PDE inhibition and increases in cGMP and cAMP on monocytic differentiation into osteoclasts, dendritic cells, and macrophages.

Keywords Bone-marrow derived macrophage · Dendritic cell · Macrophage · Monocyte · Osteoclast

1 Introduction

Monocytes are circulating peripheral immune cells that can differentiate into a number of cell types including macrophages, dendritic cells, and osteoclasts upon exposure to various cytokines (see Fig. 1). The phenotypes of these differentiated cells are highly heterogeneous and their differentiation can be affected by the cyclic nucleotides, 3'-5'-cyclic adenosine monophosphate (cAMP) and 3'-5'-cyclic guanosine monophosphate (cGMP). The intracellular levels of cAMP and cGMP are controlled through regulation of production by adenylyl and guanylyl cyclases and through degradation by cyclic nucleotide phosphodiesterases (PDEs).

Eleven different families of PDEs have been identified, and these enzymes differ in their cellular and tissue expression, substrate specificity, kinetics, modes of regulation, and sensitivity to inhibitors (Bender and Beavo 2006a). These PDEs have emerged as well-validated drug targets especially with the success of the PDE5 inhibitors Cialis, Viagra, and Levitra for the treatment of erectile dysfunction and more recently for pulmonary hypertension. In addition, several other classes of

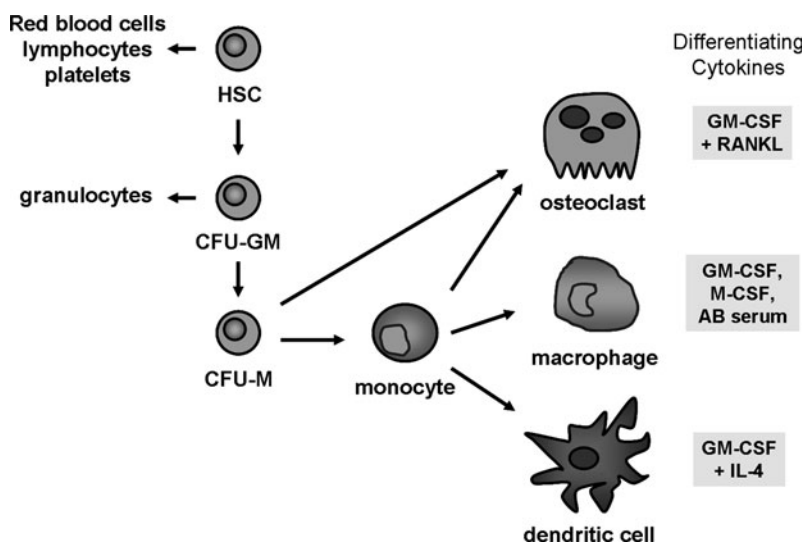


Fig. 1 Hematopoietic differentiation. Differentiation of multiple cell types from a common progenitor cell can be accomplished through their exposure to the appropriate cytokines at various stages of differentiation. Abbreviations: *HSC* Hematopoietic stem cell, *CFU* colony-forming unit, *RANKL* Receptor Activator for Nuclear Factor κ B Ligand

PDE inhibitors have been investigated as anti-inflammatory agents, anti-hypertensives, anti-depressants and as a therapy for muscular dystrophies. Of the most relevance to monocyte function and development are a number of clinical studies that have been conducted with PDE4 inhibitors as anti-inflammatory agents. While promising as *in vitro* agents and in preclinical studies, so far the PDE4 inhibitors have met with limited success as anti-inflammatory therapeutics in the clinic. Until recently, little has been known about how cyclic nucleotides and PDE inhibition can affect monocytic differentiation. Since drugs that selectively inhibit PDEs will increase cyclic nucleotide levels in targeted cells at all stages of differentiation, they are likely to affect the final phenotype of the differentiating cell as well as the differentiation process itself. PDE profiles can also change over the lifetime of the cell or during the progression of a disease state, thereby affecting its response to endogenous cyclic nucleotide-increasing agonists or to PDE inhibitor therapy (Bender et al. 2004; Essayan 2001; Gantner et al. 1997b). Indeed, in the presence of such agonists as histamine, adenosine, and prostaglandins, it is possible for macrophages to acquire a role as so-called regulatory macrophages (Mosser and Edwards 2008), while DCs are altered in their ability to activate the adaptive immune response (Morelli and Thomson 2003).

PDE profiling is a first step in analyzing the contribution of PDEs to cellular signaling pathways. In monocytes and monocyte-derived cells, PDE4 is generally considered a major PDE controlling many important inflammatory functions (Conti et al. 2003; Houslay et al. 2005; Spina 2008). However, it is important to note that PDE activity measurements can vary depending on substrate concentrations and methods used to analyze activity. PDEs also function in discrete compartments within the cell to limit the spread of the second messengers cAMP and cGMP (Bornfeldt 2006; Fischmeister et al. 2006; Houslay 2010). Therefore, a PDE whose total activity level is low could still contribute significantly to cellular function by controlling an important microdomain within the cell.

This chapter explores the role of altered cyclic nucleotide levels and inhibition of PDEs in monocytic differentiation and maturation into macrophages, dendritic cells, and osteoclasts, as well as the effect on the phenotype of the final differentiated cell.

2 Macrophage Differentiation

2.1 *HL-60*

Over 20 years ago, the HL-60 cell line was isolated from the peripheral blood of a patient with acute promyelocytic leukemia (Harris and Ralph 1985). HL-60 cells can be induced to differentiate to macrophage-like cells by a number of agents, including those that elicit a sustained elevation of intracellular cAMP or cGMP (Bang et al. 1994; Boss 1989; Nonaka et al. 1992).

Classic differentiation protocols for HL-60 cells to a myeloid cell phenotype involve treatment with either DMSO, phorbol-12-myristate-13-acetate (PMA), or all-*trans*- β -retinoic acid (RA), and this effect can be greatly potentiated by cAMP-increasing agents (Chaplinski and Niedel 1982). One group observed an effect of RA priming with pretreatment for cAMP-dependent induction of differentiation, which was not dependent on new protein synthesis (Olsson et al. 1982). Therefore, they postulated that RA priming was likely a more direct activation of existing signaling components in the cell by cAMP, presumably through PKA or Epac, or a decrease in production of an inhibitory polypeptide.

Along these lines, it was found that PMA-stimulated, cAMP-induced growth regulation is dependent on the regulatory PKA subunits PKA RI α and PKA RII β . Knockdown of the RII β subunit with an antisense oligodeoxynucleotide resulted in a decrease in cAMP-induced growth inhibition and differentiation, without affecting phorbol ester-induced differentiation (Tortora et al. 1990). Conversely, when the RI α subunit is knocked down, cell differentiation and growth arrest are increased, even in the absence of a cAMP-inducing stimulus (Tortora et al. 1991). Differentiation can also occur through stimulation of G α s-coupled receptors and increases in cAMP alone, without concomitant phorbol ester administration. Given these data, it seems that phorbol ester and cAMP-induced pathways are complementary pathways for inducing differentiation that can act synergistically given the appropriate conditions.

The differentiation of monocytes can also be affected through stimulation of G α s-coupled receptors, thereby raising intracellular cAMP. ATP can exert its effects on differentiation through stimulation of the G α s-coupled P2Y₁₁ receptor in monocytes (Boeynaems et al. 2000). The downstream effects of cAMP appeared to be mediated through PKAI, as the authors observed a decrease in differentiation using a PKA inhibitor, Rp-8-Br-cAMPS, which is more selective for PKAI than for PKAII (Jiang et al. 1997).

Cyclic nucleotide analogs are often used to dissect signaling pathways. These analogs have differing potencies and specificities, and some of the more common include hydrolysis-resistant analogs (8-Br-cAMP, 8-Br-cGMP), PKA-specific analogs (N⁶-Benzoyl cAMP) and Epac-specific analogs (8-CPT-2'-O-Me-cAMPS) (Poppe et al. 2008). These analogs can be used to stimulate downstream effectors directly. Some advantages of using analogs are that they are cell permeable, therefore easily administered to cells in culture, often nonhydrolyzable by PDEs, and the concentration can be carefully controlled. A major disadvantage is that most can stimulate all cyclic nucleotide downstream effectors and are not limited to endogenous signaling microdomains within the cell. Cho and colleagues differentiated HL-60 cells for 48 h in the presence of the cAMP analog 8-Br-cAMP and identified mature cells by their ability to produce superoxide in response to PMA (Cho et al. 2003). They concluded that cAMP induces ERK activation, and this activation is essential for differentiation. ERK activation appears to be PKA mediated as it was also not dependent on B-Raf, a downstream kinase of Rap1 and Epac. In fact, B-Raf activation was negatively regulated through PKA, and the differentiation could be inhibited by using an MEK inhibitor. While these results

were inconclusive with respect to the entire cAMP-signaling differentiation pathway, it did establish ERK as an essential player in cAMP-mediated HL-60 cell differentiation.

Beavo and colleagues performed a set of studies where chronic elevation of cyclic nucleotides was mimicked by treating HL-60 cells with 8-Br-cAMP or 8-Br-cGMP during the 3-day differentiation process in the presence of PMA (Bender and Beavo 2006b). Morphological differences were readily observed after differentiation in the presence of the cAMP analog, but 8-Br-cGMP had a minimal effect on morphology. Cell spreading in culture was inhibited in the presence of elevated cyclic nucleotides and the cells form large clumps of round cells. These differences were not attributed to any changes in macrophage function, but one could imagine that differences in “cell spreading” could be the result of differences in migration or secretion of chemotactic molecules or cytokines. Surface molecule expression was also slightly changed with treatment but differentiation was not halted. 8-Br-cAMP significantly decreased the upregulation of CD11b, a monocyte/macrophage marker, while it increased expression of the urokinase plasminogen activator (CD87); however, 8-Br-cGMP had no effect. These observed effects were mimicked by the PKA-specific activator N^6 -Benzoyl-cAMP, and therefore likely mediated through PKA.

HL-60 cells have also been used as a model system emulating human granulocyte macrophage colony-stimulating factor (GM-CSF) differentiated monocytes to determine the role of PDE1B in these cells. One variant of this isoform, the cGMP-preferring enzyme PDE1B2 (Fidock et al. 2002), is specifically upregulated upon differentiation in both GM-CSF differentiated human monocytes and PMA differentiated HL-60 cells (Bender et al. 2005). PDE1B2 activity is regulated by intracellular Ca^{2+} and may provide a link between Ca^{2+} and cAMP signaling pathways. To determine the role of PDE1B2, stable knockdown of this isoform by short hairpin RNAs was performed in differentiated HL-60 cells (Bender and Beavo 2006b). This specific isoform proved to be the major regulator of atrial natriuretic factor-stimulated cGMP levels in HL-60 cells, and the authors subsequently searched for a functional role for this newly uncovered variant. It was found that suppression of PDE1B2 expression alters some aspects of the macrophage-like phenotype, because cell spreading, phagocytic ability, and CD11b expression were augmented. Unexpectedly, the cAMP analog 8-Br-cAMP, but not 8-Br-cGMP, reversed the changes caused by PDE1B2 knockdown, indicating a role for cAMP in control of these PDE1B2-dependent functions. Moreover, despite the preference of PDE1B for cGMP as substrate, knockdown of PDE1B2 caused a decrease in basal cAMP levels, but showed no change in cGMP, and a number of PKA phosphorylation consensus sites were altered. The cause of the decrease in basal cAMP levels remains unclear. The expected increase in cGMP with PDE1B2 knockdown may be contained within localized signaling microdomains, and therefore undetectable by whole cell cGMP measurements. More likely, there is some level of crosstalk between the cGMP and cAMP signaling pathways, possibly through inhibition of an adenylyl cyclase, or activation of another cAMP-preferring PDE.

2.2 U937

The U937 cell line is a human line established from a diffuse histiocytic lymphoma and displays many monocytic characteristics (Harris and Ralph 1985). The cGMP-PDE profile of this cell line is very similar to that of a macrophage differentiated from a human CD14+ monocyte in the presence of monocyte colony-stimulating factor (M-CSF) in that it exhibits high PDE2 activity and some PDE1 activity (Bender et al. 2004). Changes in PDE4 activity have been observed in conjunction with changes in cAMP levels. Characterization of PDE4 activity in monocytic U937 cells showed that PDE4 activity can be augmented with a transient increase in cAMP of 2–4 h in length. Also, the V_{\max} of PDE4 was increased two- to threefold in the presence of salbutamol, a β_2 adrenergic agonist and the PDE4 inhibitor, rolipram, but only if cAMP levels were elevated for more than 2 h. It was determined that PDE4 activity was increased through a PKA- and protein synthesis-dependent mechanism (Torphy et al. 1992). These data imply that cAMP signaling feeds back to activate PDE4-mediated degradation of cAMP, thereby limiting the duration and magnitude of signaling through that microdomain.

Houslay and colleagues examined cAMP-PDE activity levels and found that PDE4 comprised the majority of PDE activity in U937 monocytic cells. However, upon differentiation with PMA, PDE3 activity predominated and PDE4 activity was decreased. Using antisera specific for PDE4 isoforms and RT-PCR, the investigators determined the splice variants of PDE4 that were changed during U937 differentiation to a macrophage-like cell. The N-terminal end of PDE4 isoforms contain unique isoform-specific regions that can vary in length with long, short, and supershort isoforms commonly observed. These isoforms contain variable truncations of the upstream conserved regions, or UCR domains. The long forms generally contain both UCR1 and UCR2 domains, while the short forms express only UCR2 and supershort forms merely a portion of the UCR2 domain (Houslay et al. 2005). A key distinguishing factor for these various isoforms is their susceptibility to regulation by phosphorylation in their N-terminal UCR domains. Upon differentiation to a macrophage, protein levels of the long isoform PDE4A10 and the short isoform PDE4B2 were markedly increased, whereas long-form PDE4A4 remains unchanged, and short forms PDE4D3 and PDE4D5 were downregulated (Shepherd et al. 2004). Additionally, it was found that phospho-ERK was increased with acute PMA challenge, and this challenge gave a time-dependent inhibition of PDE4 activity in U937 monocytes and an increase in PDE4 activity in U937 macrophages, both of which are dependent on ERK activation. The differential effects of ERK activation can be directly attributed to the remodeling of the pattern of PDE4 isoform expression. The predominant PDE4 activity in monocytes is contributed by long PDE4D isoforms, whereas in macrophages PDE4B short form activity is the major source of PDE4 activity. This remodeling has functional consequences since PDE4D long isoforms will become inhibited, PDE4B short forms will be activated upon phosphorylation by ERK, and PDE4A isoforms are unaffected as

they do not contain ERK phosphorylation sites (Baillie et al. 2000). This presents an attractive therapeutic possibility if PDE4 isoform-selective inhibitors can be developed, allowing the differential regulation of monocytes and macrophages.

U-937 differentiation can be induced by increasing cAMP levels, either using PGE₂, the adenylyl cyclase agonist forskolin, or a nonhydrolyzable cAMP analog, db-cAMP (Brodsky et al. 1998; Shayo et al. 1997). Surprisingly, activation of the G α s-coupled histamine receptor is not able to induce U937 differentiation, despite its ability to activate adenylyl cyclase and cause a transient increase in intracellular cAMP (Shayo et al. 1997). Upon further investigation, it was discovered that the histamine receptor rapidly becomes desensitized through a GRK2-dependent mechanism and that a sustained elevation of cAMP is required for induction of differentiation (Fernandez et al. 2002; Legnazzi et al. 2000; Shayo et al. 2001). The stable overexpression of the histamine H2 receptor (H2R) alone also induced differentiation (Monczor et al. 2006). The increased expression of H2R leads to an increase in basal cAMP levels and a leftward shift of the dose–response curve. There was a maximal response to agonist similar to those cells expressing normal amounts of H2R, indicating that overexpression of H2Rs results in increased numbers of functional spare receptors in this system. In addition to the generation of spare receptors, there was a compensatory increase in GRK2 expression and PDE activity stimulation. Despite the onset of these regulatory mechanisms, an H2 agonist and the PDE4 inhibitor, rolipram, were still able to induce differentiation in these cells, contrary to the previous observations in naïve cells (Monczor et al. 2006).

The time course of cAMP elevation was determined to be critical for the induction of U937 differentiation. Using the cAMP-elevating agonists amthamine (a histamine H2 receptor agonist), PGE₂ (an EP receptor agonist), and forskolin (an adenylyl cyclase agonist), Shayo and colleagues were able to demonstrate a time dependence to cAMP-induced differentiation in U937 cells (Shayo et al. 2004). Amthamine caused a transient increase in cAMP returning to baseline in about 3 h, whereas forskolin and PGE₂ created a more prolonged elevation of cAMP, which was still sustained at the final measurement of 4 h. This prolonged cAMP elevation was necessary for differentiation of U937 cells, as indicated through the observed change in differentiation markers such as an increase in C5aR expression, arrested proliferation, and a decrease in c-myc protein levels. The mechanism of this discrepancy seems to be H2R dependent occurring through GRK2-mediated desensitization of the receptor. PGE₂ and forskolin bypass H2R and therefore are able to sustain cAMP elevation. Homologous desensitization of the EP receptor by PGE₂ was evident immediately upon stimulation and persisted for 40 min. However, a sustained cAMP elevation via either PGE₂ or forskolin can also induce heterologous desensitization of the H2 receptor in a PKA/PKC-dependent manner. The data also showed a transient upregulation of c-Fos with amthamine and sustained c-Fos expression with PGE₂ and forskolin. This correlated well with the fact that sustained c-fos expression is known to be essential for U937 differentiation. Overall, these studies indicate that cAMP levels must be sustained long term to induce U937 differentiation.

2.3 *Human AB Serum and M-CSF*

2.3.1 **AB Serum**

Human peripheral blood monocytes can be differentiated to macrophages in culture in human AB serum, and enriched through their adherence to tissue culture plates. Initially, the monocytes contain mostly PDE4 activity, with some PDE3 activity. As the cells differentiate in culture, the predominate PDEs emerge as PDE1 and PDE3, with rapidly declining amounts of PDE4 activity. PDE2 and PDE5 levels remain consistently low throughout the differentiation process (Gantner et al. 1997a). In another study with human monocytes, the majority of the PDE4 activity was found in the soluble fraction, and the PDE3 activity was associated with the particulate fraction similar to its location in many other tissues (Verghese et al. 1995). AB serum-differentiated monocytes exhibit a PDE profile strongly resembling that observed for human alveolar macrophages. A comparison of TNF- α production elicited by lipopolysaccharide (LPS) between monocytes and monocyte-derived macrophages showed inhibition of TNF- α production with increases in cAMP brought on by PDE4 inhibition in both cell types (Gantner et al. 1997a). PDE4 controlled the largest portion of TNF- α production in monocytes, as about 80% could be blocked with a PDE4 inhibitor, with PDE3 inhibition decreasing TNF- α production approximately 10%. In macrophages, PDE3 and PDE4 controlled more equal amounts, but PDE inhibition was only effective when administered in the presence of an additional cAMP stimulus, perhaps indicating a lower cAMP tone in macrophages than monocytes.

Human monocytes cultured in human AB serum also have a very similar PDE profile to that present in the cell line Mono-Mac-6. Using this cell line as a surrogate, a closer look at the changes in the PDE4 isoform levels during challenge with cAMP-increasing agents showed that db-cAMP, PGE₁, or LPS transiently increased PDE4A, B, and D mRNA and protein levels (Verghese et al. 1995). However, the time course and magnitude of induction of each isoform was not identical. PDE4B levels rose early, mRNA increased from 2 to 4 h, protein increased from 2 to 8 h; with PDE4A and PDE4D rising later in the time course, mRNA increased from 2 to 8 h and protein increased after 5–24 h of treatment. This increase was specific for PDE4 isoforms as there was no concomitant increase in PDE3 activity with differentiation. An additional level of regulation was observed for the PDE4 isoforms as differences in the induction of mRNA when compared to protein levels were apparent over the time courses tested, suggesting that the PDE4 isoforms and splice variants were transcriptionally regulated.

2.3.2 **M-CSF + AB Serum**

While PDE activities, and therefore the duration of cAMP and cGMP signals, can change during differentiation, the levels of their downstream signaling effector

proteins can also be altered. The immediate downstream mediators for cAMP are PKA, Epac, and nucleotide-gated ion channels. The levels of these mediators can also change over the course of monocyte to macrophage differentiation and maturation. Taskén's group identified a number of signaling molecules downstream of cAMP in monocytes and macrophages. Monocytes contained the regulatory PKA subunits RI- α , RII- α and the catalytic C subunit, with high levels of RI- α and lower levels of RII- α (Bryn et al. 2006). Monocytes differentiated in the presence of human AB serum and M-CSF produce macrophages that exhibit a threefold increase in Epac1 mRNA levels. Presumably, this would translate into an increased role for Epac and its downstream signaling through Rap1 and the MAP kinase pathways in macrophage function. Strangely, these investigators found that Epac activation had a minimal effect on mature macrophage functions for most conditions tested, such as inhibition of TNF- α or IL-12 secretion. The majority of macrophage functions remained PKA-dependent and therefore unaffected by treatment with the Epac-specific activator, 8-CPT-2'-O-Me-cAMP. In one functional assay for phagocytic capability, however, FcR-mediated phagocytosis was reduced equally by both PKA and Epac-specific activators in mature macrophages, while in undifferentiated monocytes, there was no effect of the Epac-specific cAMP analog alone. Although this study was not exhaustive, it definitively showed a minimal involvement of Epac in the regulation of monocyte/macrophage inflammatory function and a major role for PKA.

2.3.3 M-CSF

When monocytes are instead differentiated using M-CSF and fetal calf serum, a distinct set of characteristics are acquired (Geissmann et al. 2010). These cells have moderate levels of a number of PDEs involved in degradation of both cGMP and cAMP and changes in guanylyl cyclase activity. M-CSF-derived macrophages retain modest PDE4, PDE3, and PDE1 levels. For the cGMP PDEs, PDE2 activity increases slightly with M-CSF differentiation, and PDE5 activity decreases to undetectable levels (Bender and Beavo 2004; Bender et al. 2004). In addition to the regulation of the cyclic nucleotide-degrading PDEs, these investigators also observed changes in the levels of the cGMP-producing guanylyl cyclases, likely affecting the availability of cGMP in the cells. The levels of GC-A were undetectable in monocytes, with slightly increased levels in M-CSF macrophages, while soluble GC mRNA levels were dramatically downregulated upon differentiation to a macrophage.

The physiological implications of these changes are not immediately clear. Expression of particular PDEs or cyclases is often linked with specific signal transduction pathways through localization with a specific receptor, anchoring protein or kinase. For example, if PDE5 is tightly linked with other members of the cGMP pathway, the loss of PDE5 with M-CSF differentiation may be coupled to a loss in sGC and a decrease in responsiveness to NO (Bender et al. 2004). Similar correlations could also occur with cAMP signaling pathways, leading to

changes in cAMP-PDEs, PKA, Epac, adenylyl cyclases, or any number of downstream signaling components.

2.4 GM-CSF

Monocyte to macrophage differentiation can also occur when monocytes are exposed to the cytokine GM-CSF (Geissmann et al. 2010). These macrophages have a phenotype similar to that observed for alveolar macrophages. The cGMP-PDE profile in CD14⁺ monocytes isolated from human blood consists of mostly PDE5 activity, with some PDE1 and PDE2 activity and low PDE3 and modest PDE4. Upon differentiation for 7 days in GM-CSF, PDE1 emerges as the predominant cGMP-PDE present in these macrophages, consisting mostly of the PDE1B isoform and the cAMP-PDE activity was attributed equally to PDE3 and PDE4 (Bender and Beavo 2004; Bender et al. 2004; Hertz et al. 2009a). Also characterized in these studies was the cGMP-PDE composition of the monocytic cell line THP-1 which was found to be similar to GM-CSF-derived macrophages upon differentiation with PMA. THP-1 is a human acute monocytic leukemia cell line with high levels of PDE1B, but very low levels of PDE1A and PDE1C and very little PDE2 activity. These cells can be differentiated to a macrophage-like cell upon stimulation with PMA. While these THP-1-derived macrophages contained high levels of PDE1B, the levels of all PDEs were consistently high in the undifferentiated monocyte and throughout differentiation, in contrast to CD14⁺ monocyte-derived macrophages (Bender et al. 2004).

Upon further investigation, it was discovered that a unique splice variant, PDE1B2 (Fidock et al. 2002), is the form upregulated upon monocyte to macrophage differentiation in the presence of GM-CSF (Bender et al. 2005). Interestingly, this isoform of PDE1B has a threefold lower EC₅₀ for calmodulin than its counterpart PDE1B1. This calmodulin-sensitive PDE has a separate transcriptional start site that can be activated by monocytic differentiation to a macrophage, but is suppressed upon the addition of IL-4 and subsequent differentiation of the monocyte to a dendritic cell. The authors postulate that selective upregulation of a calcium/calmodulin-sensitive cGMP-preferring PDE could provide a mechanism by which calcium transients can control the duration and amplitude of the cGMP signal.

The cAMP-PDE activities in GM-CSF-differentiated monocytes were determined using a PDE activity assay, with PDE4 showing the largest amount of activity in monocytes, with decreasing amounts of activity during differentiation. However, in fully differentiated macrophages PDE3 and PDE4 comprised equivalent amounts of cAMP PDE activity (Gantner et al. 1997a, b, 1999; Hertz et al. 2009a). It should also be noted that PDE1B is a dual specificity PDE, able to hydrolyze both cAMP and cGMP. Although it prefers to hydrolyze cGMP over cAMP, a moderate amount of cAMP-PDE activity is contributed by PDE1B in these cells.

The activity and type of cyclase present is another factor that can affect cyclic nucleotide levels in any cell. CD14+ monocytes were found to have high levels of soluble guanylyl cyclase, which was decreased slightly upon differentiation to a macrophage with GM-CSF (Bender et al. 2004). The levels of GC-A were undetectable in monocytes, with greatly increased levels in macrophages. These changes could affect the cells' ability to respond to a number of cGMP-increasing factors including nitric oxide and atrial natriuretic peptide (ANP).

More recently, the effects of chronic elevated cyclic nucleotide levels on the function and final phenotype of monocytes differentiating to alveolar-type macrophages were investigated (Hertz et al. 2009b). Human CD14+ monocytes were differentiated in the presence of GM-CSF for 6 days in combination with cAMP-increasing agents, such as the adenylyl cyclase agonist forskolin, the EP receptor agonist PGE₂, and selective PDE inhibitors to assess the effects of elevated cAMP levels on the final phenotype of the macrophage. Initially, monocytes were treated with a high dose of forskolin in culture for 6 days. A microarray analysis was performed on RNA extracted from the resulting macrophages and, unexpectedly, a number of proinflammatory genes were upregulated, especially chemokines binding to the CXCR2 and CCR2 classes of receptors. Expression of the cytokines IL-10 and IL-6, as well as a number of genes involved in wound-healing, were also increased upon differentiation in the presence of cAMP (see Fig. 2a). A phenotypic analysis of these forskolin-treated cells showed that differentiation was not halted as was seen previously with GM-CSF + IL-4-induced dendritic cell differentiation (Giordano et al. 2003), but the expression of a number of surface markers was altered. Notably, the downregulation of CD14, the coreceptor for LPS, and CD163, a scavenger receptor, was prevented with forskolin treatment.

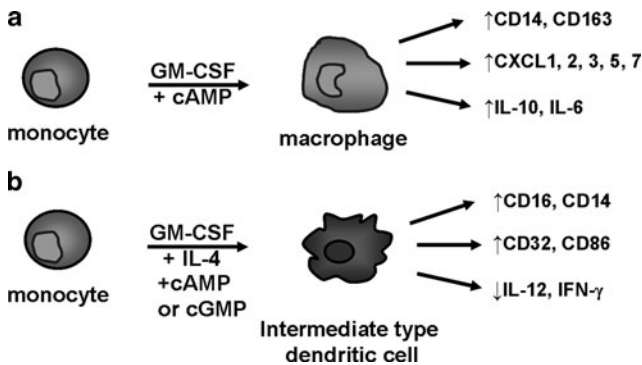


Fig. 2 Effects of cAMP on monocytic differentiation to macrophages and dendritic cells. Monocytes isolated from human blood can be differentiated in the presence of cytokines GM-CSF or GM-CSF plus IL-4 to give rise to macrophages and dendritic cells, respectively. When differentiated in the presence of elevated cyclic nucleotides, the final phenotypes of these cells are altered. Differences in cytokine and chemokine production are observed as well as changes in surface marker expression levels

To determine which PDEs were controlling the pools of cAMP regulating surface marker and chemokine expression, selective inhibitors of PDE2, 3, and 4 were used in combination with a low dose of forskolin. The low dose of forskolin showed a minimal change in mRNA and protein levels of these genes (Hertz et al. 2009a). However, when that same dose of forskolin was administered with a PDE inhibitor it caused a large response. This is exactly what would be predicted for a synergistic effect of PDE inhibition coupled with adenylyl cyclase activation. Using this technique, it was determined that PDE4 primarily controls the signaling microdomain regulating expression of surface markers and chemokines.

The intracellular signaling pathways involved in regulation of these chemokines seem to be largely downstream of Epac, not PKA as seen for cAMP's effect on most of the proinflammatory cytokines (Hertz et al. 2009a). Cyclic nucleotide analogs which specifically activate Epac induce chemokine expression at both the mRNA and the protein levels, while a PKA-specific activator had a minimal effect on chemokine levels. Downstream signaling pathways leading to changes in macrophage inflammatory gene expression are generally regulated through the canonical NF- κ B pathway. Chemokine production was attenuated with a general inhibitor of the NF- κ B signaling pathway, the IKK1/2 inhibitor BMS-345541, but surprisingly remained elevated after treatment with a peptide inhibitor of the canonical p50/p65 NF- κ B heterodimer. While inflammatory cytokine production is normally regulated through the p50/65 NF- κ B pathway in macrophages, it seems that production of these chemokines via elevated cAMP may occur through the noncanonical p50/p105 NF- κ B pathway. Also affecting this pathway was a negative regulator of NF- κ B, the transcription factor ATF3, which exhibits both reduced mRNA levels and attenuated binding to the CXCL7 promoter when forskolin is present during differentiation (Hertz et al. 2009a). The relief of inhibition of ATF3 on promoter regions containing NF- κ B would promote expression of many inflammatory genes (Gilchrist et al. 2006).

The signaling pathways downstream of cAMP can also be changed during differentiation. Human monocytes stimulated with LPS produce large amounts of TNF- α , IL-6, and IL-8. The release of these chemokines can be reduced with a p38 kinase inhibitor. However, in macrophages differentiated in GM-CSF for 12 days, p38 inhibition has a lesser effect on production of these cytokines while TNF- α production is effectively blocked with an ERK inhibitor (Tudhope et al. 2008). This suggests that kinase usage can also change as a monocyte differentiates to a macrophage, with monocytes favoring p38 kinase and macrophages preferring ERK to control cytokine production.

3 Dendritic Cell Differentiation

Monocytes differentiated in the presence of GM-CSF + IL-4 gain a dendritic cell phenotype (Geissmann et al. 2010). The PDE profile of these dendritic cells (DCs) showed lower PDE4 levels than monocytes, increased PDE3, and a moderate

upregulation of PDE1B (Gantner et al. 1999; Giordano et al. 2003). Regarding PDE4 specifically, PDE4B was found to be expressed at high levels in monocytes, with PDE4A being the predominant PDE4 isoform in DCs (Heystek et al. 2003).

Elevation of cyclic nucleotides through administration of PGE₂ has been shown to enhance the differentiation and maturation of DCs under serum-free cell culture conditions. In lieu of fetal calf serum, a monocyte conditioned medium was used to culture the monocytes in the presence of GM-CSF and IL-4 and the addition of PGE₂ greatly enhanced their differentiation (Jonuleit et al. 1997). It was hypothesized that preexposure of monocytes and DCs to cAMP-elevating agents, such as PGE₂, in tissues could have functional consequences for the final phenotype of the cell and the subsequent priming of naïve T cells in lymph nodes.

In other studies to test the effects of elevated cAMP on DC function, monocytes were isolated from human peripheral blood and differentiated for 6 days in the presence of fetal calf serum, GM-CSF and IL-4 in the presence or absence of PGE₂ (Kalinski et al. 1997) or with the addition of the adenylyl cyclase activator forskolin, cyclic nucleotide analogs (8-Br-cGMP and 8-Br-cAMP) or the nonselective PDE inhibitor, isobutylmethylxanthine (IBMX) (Giordano et al. 2003). The phenotype of this cAMP-generated DC was characterized by analyzing its surface marker and cytokine expression profile to determine whether the cell was directed to a DC, or another cell type. (see Fig. 2b) Normally during monocytic differentiation to a DC, the surface markers CD14 and CD1a are reciprocally regulated with the cells showing a downregulation of the monocytic marker CD14 and an upregulation of DC marker CD1a. The reciprocal regulation of these surface markers was prevented with increased cyclic nucleotides (Giordano et al. 2003). However, cyclic nucleotides do not seem to affect the regulation of other markers such as DC-SIGN, a DC-restricted C-type lectin involved in the early interaction between DCs and naïve T cells and also DC trafficking and internalization of Ags, or the downregulation of the high affinity IgG-binding receptor, CD64/FcγRI. Terminal differentiation of myeloid cells is generally characterized by a switch from CD64/FcγRI to the low affinity CD32/FcγRII. Interestingly, CD32/FcγRII expression was not blocked by elevated cyclic nucleotides, but was slightly enhanced and the final phenotype was more reminiscent of the levels seen on macrophages (Giordano et al. 2003). The regulation of the costimulatory molecules CD80 and CD86 was unaffected by PGE₂ (Kalinski et al. 1997) but CD86 and MHC class II were upregulated with cAMP analog treatment over the 6-day differentiation process and the reason for this is unclear (Giordano et al. 2003). The authors concluded that DC differentiation was halted at an intermediate cell stage between monocytes, macrophages, and DCs.

This intermediate population of cells was not homogeneous; however, since differentiation in the presence of elevated cyclic nucleotides also upregulated a subset of cells expressing CD16 (Giordano et al. 2003), a surface marker upregulated in some pathological conditions such as HIV and autoimmune diseases (Grage-Griebenow et al. 2001). CD16 is expressed on less than 10% of circulating monocytes and is generally expressed on the cells that are CD14^{lo}. After cyclic nucleotide treatment *in vitro*, the percentage of CD16⁺ cells increased dramatically,

up to as much as 50% of the total cell population (Giordano et al. 2003). The authors speculate that cyclic nucleotide signaling may be a part of the intracellular mechanisms used to modulate expansion of this subpopulation in pathological conditions.

DCs generated in the presence of physiological concentrations of PGE₂, and therefore elevated cAMP, demonstrated Th-cell stimulation abilities comparable to the control DCs differentiated in the absence of PGE₂. However, the ability of PGE₂-DCs to produce IL-12 was markedly reduced when stimulated with IL-12-inducing agents such as soluble CD40L, and even in the presence of the normally potent IL-12 inducing agent, IFN- γ (Kalinski et al. 1997). When the concentration of PGE₂ was increased to 100 nM, the cells' IL-12 producing ability was completely abolished. Conversely, production of the anti-inflammatory cytokine IL-10 was markedly increased with PGE₂ pretreatment, but decreased with IFN- γ cotreatment. This profile was maintained over 48 h after the removal of PGE₂, and the ability of these DCs to produce other Th cell-polarizing cytokines was also altered. PGE₂-DCs retained higher IL-4 and IL-5 producing capacity and produced 70% less IFN- γ than control DCs. The ability of PGE₂-DCs to promote Th2-type differentiation of naïve T cells was largely due to the absence of IL-12, which would normally drive the cell to a Th1 phenotype, not due to the presence of an additional secreted factor. Similar results were observed by another group when a PDE4 inhibitor was administered during differentiation of monocytes to dendritic cells, prior to final maturation (Heystek et al. 2003).

In a pathological situation, cyclic nucleotide levels will be elevated not only during differentiation of monocytes, but also during maturation into the final macrophage or dendritic cell. The effects of PGE₂ on DC maturation were found to act as a cofactor and synergize with TNF- α and IL-1 β to induce final maturation of immature DCs (Kalinski et al. 1998). The adenylyl cyclase agonist, forskolin, mimicked the effects of PGE₂, indicating that final maturation can be influenced directly by cAMP levels. After final DC maturation, PGE₂ and IL-10 were no longer able to affect the phenotype of the DC, indicating that shaping of the mature DC phenotype occurs while the immature DC is a tissue resident and capable of taking up antigen. Therefore, the levels of IL-12 production and mature DC phenotype are predetermined at the stage of immature DC in peripheral tissues. The stability of the phenotype for up to 2 days after exposure to PGE₂ implies that the DC phenotype will be retained upon its arrival in the draining lymph node, allowing it to contribute to the development of Th2-biased responses without picking up irrelevant signals as it migrates to the lymph node.

This effect on maturation is not isolated only to cAMP, similar effects have been observed with elevated intracellular levels of cGMP. In one set of studies, mature DCs were derived from CD14+ human monocytes and matured/activated with LPS (Morita et al. 2003). When these cells were pretreated with the guanylyl cyclase agonist ANP, the LPS-induced expression of IL-12 and TNF- α was decreased and IL-10 expression was augmented similar to what was observed for cAMP (Morita et al. 2003). This phenotype is also able to polarize CD4+ T cells toward promoting Th2 responses through interactions in the absence of IL-12 production.

DCs can mature upon exposure to LPS and acquire a distinct set of surface markers that are regulated by cyclic nucleotides. Cyclic nucleotide treatment inhibited the increase in DC-LAMP and CD83 normally brought on with LPS stimulation. DC-LAMP is a lysosome-associated membrane glycoprotein that belongs to DC-specific apparatus for antigen processing. Different antigen-presenting cells have distinct endocytic routes used for processing antigen, with specific markers characterizing each step. CD68 or macrophage mannose receptor is another lysosomal marker expressed on both macrophages and DCs and is inversely regulated from DC-LAMP; as DC-LAMP appears in these compartments, CD68 levels are reduced. Curiously, while DC-LAMP expression was reduced in the presence of cyclic nucleotides, CD68 remained unaffected. Therefore, it seems that an increase in cyclic nucleotide levels during differentiation could impair the ability of DCs to process antigen, but does not inhibit antigen-processing machinery shared with other cells of the myeloid lineage.

DCs differentiated in the absence of PGE₂, but matured in its presence, also acquire an increased ability to migrate toward the lymph nodes via CCR7 and exhibit improved ability to activate CD4 T cells suggesting the involvement of PGE₂ in facilitating CCR7 signaling (Luft et al. 2002; Scandella et al. 2002). However, when DCs are differentiated in the presence of cyclic nucleotide analogs, the induction of CCR7 by LPS is reduced (Giordano et al. 2003). CCR7 confers the ability onto dendritic cells to migrate toward CCL19, which is a vital step in leading DCs to lymph nodes and downstream T-cell activation. Despite a potential reduction in CCR7 levels, these DCs do still acquire strong antigen-presenting capabilities, as they are able to activate CD4 T cells in allogenic mixed leukocyte reactions, a measure of their function as DCs. This is likely due to the upregulation of the costimulatory molecule CD86 and the antigen-presenting molecules MHCII DQ and DR (Giordano et al. 2003). This effect of cyclic nucleotides seems to be dependent on the timing of exposure to cAMP-inducing agents during differentiation or maturation, as somewhat disparate results are obtained depending on the methodology used for differentiation, maturation, and cyclic nucleotide elevation.

Interestingly, dendritic cell maturation in serum-free conditions, but in the presence of a cytokine cocktail, leads to a DC capable of a nonbiased Th2 response (Morelli and Thomson 2003; Scandella et al. 2002). While this is in contrast with previous *in vitro* data using fetal calf serum-containing media, it is likely that in the context of an inflammatory response *in vivo* the effect of PGE₂ is to permit the generation of DCs that are capable of secreting the full range of cytokines, not just Th2 cytokines.

Stimulation of adenosine receptors and subsequent elevation of cAMP during monocyte to DC differentiation results in a distinct cell population characterized by changes in expression of cell surface markers common to both DCs and monocyte/macrophages, but remaining morphologically similar to DCs (Haskó et al. 2009). Using knockout animals and pharmacologic inhibition of adenosine receptors, it was discovered that the G α s-coupled A_{2B} adenosine receptor was the mediator of the phenotypic observations. Adenosine-differentiated DCs express high levels of angiogenic (VEGF), proinflammatory (IL-8, COX-2, IL-6), immune suppressive

(IL-10, TGF- β), and tolerogenic factors (indoleamine 2,3-dioxygenase) (Novitskiy et al. 2008). Consequently, these cells are impaired in their ability to induce T-cell proliferation, by rendering the T cells anergic with indoleamine 2,3-dioxygenase, and also exhibited a reduced capacity for IFN- γ production. As expected, these phenotypic changes result in drastic alterations in the capacity of these cells to respond properly to abnormalities or inflammatory stimuli. For example, if these adenosine-differentiated DCs were injected directly into a subcutaneous tumor in mice, they actually promoted tumor growth through increased angiogenesis in the tumor, rather than resolving the abnormality (Novitskiy et al. 2008).

The role of the A_{2B} receptor in cAMP-mediated regulation of pathological conditions and cell differentiation has been historically difficult to study due to its low affinity for adenosine and other selective agonists. A_{2B} receptors are stimulated by micromolar concentrations of adenosine, rather than the normal physiological submicromolar levels commonly observed and associated with the A_1 , A_{2A} , and A_3 receptors. However, this low affinity may carry physiological importance as high concentrations of adenosine are often found at the site of traumatic injury, ischemia, or inflammation (Haskó et al. 2009). Overall, DC differentiation and maturation is retarded in the presence of elevated cyclic nucleotides, and this results in altered phenotypic characteristics affecting many key DC functions.

4 Osteoclast Differentiation

Osteoclasts are a unique cell type involved in calcium homeostasis in the body. They perform this function through the resorption of bone in response to either direct or indirect hormonal regulation by parathyroid hormone, calcitonin, and 1,25-dihydroxyvitamin D₃ (Bar-Shavit 2007; Nakashima and Takayanagi 2009). Osteoclasts work in harmony with their bone-building counterparts, osteoblasts, to maintain the level of free calcium required for many bodily functions. Osteoblasts regulate formation and differentiation of osteoclasts and so are major players in homeostatic control of bone remodeling. cAMP and PDEs have profound effects on osteoblastic function, namely the regulation of receptor activator of nuclear factor-kappa B ligand (RANKL) production, but this will not be discussed in depth in this chapter, as osteoblasts are derived from the mesenchymal lineage, not the hematopoietic/monocytic lineage.

Osteoclasts are derived from the hematopoietic lineage with many steps along their differentiation pathway, but require only two major cytokines to complete their maturation. First, the hematopoietic stem cell can be started along the osteoclastic pathway by responding to cytokines such as M-CSF to become an osteoclast precursor cell or CFU-M (see Fig. 1). These cells are monocyte-like and can be differentiated to macrophages or dendritic cells at this stage, if they do not receive additional signals guiding them toward an osteoclast. This major driving signal for

osteoclast formation is the RANKL cytokine produced by osteoblasts in response to hormonal stimulation. RANKL causes the CFU-M to commit to becoming an osteoclast and soon becomes a mononucleated osteoclast. Osteoclasts must fuse together and become multinucleated (three or more nuclei) and fully mature before they are able to perform their bone-resorbing functions (Bar-Shavit 2007; Nakashima and Takayanagi 2009).

Elevated cAMP, increased through treatment with PGE₂, has been reported to both increase and decrease osteoclast formation. These discrepancies seem to be attributable to a number of factors: the dose of PGE₂ being administered, the timing of PGE₂ administration, the type of cells being studied, and the methods used to prepare osteoclasts and/or osteoblasts. It has been demonstrated that addition of a cAMP-elevating agonist such as PGE₂ or forskolin or an inhibition of PDE activity can increase osteoclast formation from either human or mouse bone marrow cultures. Several groups have reported an increase in osteoclast formation from bone marrow with PGE₂ (Fujita et al. 2003; Noh et al. 2009; Take et al. 2005; Wani et al. 1999), 8-isoprostaglandin E2 (an oxidized form of PGE₂) (Tintut et al. 2002), forskolin (Ransjö et al. 1999), a cAMP analog (Wani et al. 1999), or PDE inhibition (Noh et al. 2009; Park and Yim 2007; Takami et al. 2005; Yamagami et al. 2003). This increase in osteoclast number and function can derive from two separate mechanisms: the stimulation of osteoblasts to produce RANKL, and direct effects on osteoclast precursors.

The concentration of PGE₂ is vitally important in the regulation of osteoclast differentiation from bone marrow. Moderate doses of PGE₂, ranging from 1 to 100 nM, seem to stimulate osteoclastogenesis in adherent cell-depleted hematopoietic monocultures in the presence of M-CSF and RANKL for both mouse and human bone marrow cells (Fujita et al. 2003; Ono et al. 2005). One group reported a synergistic increase in osteoclast formation when stimulated with RANKL and 1 μM PGE₂ in bone marrow cultures (Wani et al. 1999). However, several groups report that when the concentration of PGE₂ is 1 μM or higher, osteoclastogenesis is inhibited and subsequent bone resorption is decreased (Fujita et al. 2003; Ono et al. 2005; Park and Yim 2007). This does not hold true for all cell types as in RAW 264.7 cells, a mouse monocyte-like cell line, PGE₂ concentrations at 1 μM stimulate osteoclast formation through PKA-dependent phosphorylation of TAK1 (Kobayashi et al. 2005a).

A second model of osteoclast generation uses CD14⁺ monocytes from human blood differentiated in the presence of M-CSF and RANKL to form osteoclasts. This monocyte to osteoclast transformation is inhibited by PGE₂ at all concentrations of PGE₂ tested, 0.1–100 nM (Itonaga et al. 1999; Take et al. 2005). However, it was found that the timing of administration of PGE₂ also played a critical role in the ability of osteoclast precursors to differentiate into osteoclasts. If PGE₂ was added at days 0–2 of culture, differentiation was prevented, if added later in the maturation process at days 6–8 there was no change in the number of mature osteoclasts after 8 days (Take et al. 2005). Similarly, the differentiation of bone marrow cells was stimulated when PGE₂ was present during days 0–2 of culture, but no change was observed when added at days 6–8.

In order for the cells to be responsive to PGE₂ levels, they must carry the appropriate receptors. The prostaglandin receptors are EP1, 2, 3, and 4 coupled to G α _q, G α _s, G α _i, and G α _s, respectively. The high level expression of the prostaglandin receptors EP2 and EP4 in precursor cells allows the cells to be responsive to stimuli like PGE₂ and produce cAMP through adenylyl cyclase stimulation. Upon differentiation, the prostaglandin receptors are downregulated, preventing the stimulation or inhibition of the mature osteoclast by PGE₂ (Kobayashi et al. 2005b; Take et al. 2005). If EP4 is reintroduced using an adenoviral transfection system, osteoclastic function was drastically inhibited (Kobayashi et al. 2005b). PGE₂ also can directly inhibit bone resorbing function in mature OCs thru EP4 (Mano et al. 2000) and an EP4 antagonist inhibited osteoclast formation (Ono et al. 1998). EP1 and EP3 do not seem to play a major role in osteoclast formation because no increase in osteoclast formation was observed with the administration of butaprost, an EP1/3 agonist, while EP2-selective agonists slightly increase osteoclast formation (Ono et al. 1998). The G α _s-coupled prostaglandin receptors EP2 and EP4 seem to have nonredundant roles in osteoclast formation as EP2 knockout mice exhibit impaired osteoclast development, likely due to both a defect in the osteoblast and having direct effects on PGE₂-stimulated osteoclast function (Li et al. 2000).

PDEs also play a role in controlling cAMP levels during differentiation of bone marrow precursors to osteoclasts. Inhibition of PDE4 with rolipram indirectly stimulates osteoclast formation through the ERK, p38, MAP kinase pathways by leading to a change in COX2 expression in osteoblasts. Elevated COX2 expression leads to an increase in PGE₂, which in turn increased RANKL production by the osteoblasts (Cho et al. 2004; Park et al. 2007; Takami et al. 2005). PDE4 inhibition can also have effects directly on osteoclast formation from their precursors. Yamagami and colleagues showed that when the PDE4 inhibitor XT-611 was added to either a culture of bone marrow-derived osteoclastic precursors, or a coculture with osteoblasts, osteoclast formation was blocked when given concomitantly with PGE₂. This effect seemed to be specific for PDE4 inhibition, as inhibition with either a PDE3-selective inhibitor milrinone, or the cGMP-PDE inhibitor zaprinast, had no effect on osteoclast differentiation in the presence of PGE₂ (Yamagami et al. 2003). In a similar study, it was found that both PDE3 and PDE4 inhibitors had an effect on osteoclastic differentiation; however, these effects were largely on the osteoblasts, not on the osteoclasts themselves (Noh et al. 2009). PDE4 inhibitors can also potentiate the effects of endogenous agonists such as calcitonin. Calcitonin acts directly on osteoclasts to decrease osteoclastogenesis and this process was found to be PKA dependent and rolipram potentiated these effects (Miyamoto et al. 2006).

PDE4 inhibitors as a therapeutic intervention for osteoporosis show some promise based on animal models of osteopenia. Additionally, single-nucleotide polymorphisms in the PDE4D gene have been shown to correlate with variations in bone mineral density, possibly implicating PDE4D as a contributing genetic factor in human osteoporosis (Reneland et al. 2005). Both rolipram and pentoxifylline were reported to increase bone density in normal mice after 5 weeks of treatment (Kinoshita et al. 2000). In rats, a PDE4 inhibitor decreased bone loss in

a model of osteoporosis in rats (Miyamoto et al. 1997) and increased bone density in ovariectomized, neurectomized rats (Waki et al. 1999).

One report regarding cGMP and osteoclastogenesis suggests that cGMP can block the differentiation of osteoclasts, but only when present during the final 3 days of a 6-day differentiation process (Holliday et al. 1997). As there has been some debate as to the pathways used for intracellular signaling by nitric oxide, both nitric oxide releasers such as sodium nitroprusside, used at low concentrations, and the cGMP analogues 8-Br-cGMP and dibutryl-cGMP, were used to demonstrate reduced osteoclast formation in the presence of elevated cGMP. Elevating cGMP using an inhibitor of PDE5, zaprinast, also inhibited osteoclast formation when administered through the final 3 days of differentiation, while decreasing cGMP using nitric oxide synthase inhibitors increased osteoclast formation. It was concluded that elevated cGMP inhibits osteoclast formation, but only when present during the final stages of differentiation.

5 Murine Bone Marrow Differentiation

Bone marrow progenitors can be differentiated to macrophages in the presence of a number of factors, such as M-CSF, GM-CSF, and PMA. It has long been observed that agents that can elevate cAMP can inhibit macrophage formation from progenitors. M-CSF can also induce proliferation of differentiated bone marrow macrophages, and PGE₂ negatively regulates this process (Kurland et al. 1977, 1978a, b). Several agents that can increase cAMP, such as PGE₂, 8-Br-cAMP, and IBMX, can suppress the mitogenic actions of M-CSF, GM-CSF, and PMA through the inhibition of DNA synthesis. This effect of cAMP does not appear to affect the cells' early response to colony-stimulating factors, but exerts its effect during the late G1 phase in the cell cycle (Vairo et al. 1990). Inhibition by PGE₂ seems to be part of a negative feedback loop controlling proliferation of monocytes and macrophages. PGE₂ may feedback to control macrophage formation by inhibiting proliferation and differentiation of immature monocytoid cells.

M-CSF-induced proliferation of mouse bone marrow macrophages is suppressed by adenosine-mediated signaling through the G α s-coupled A_{2B} receptors. The downstream cAMP signaling mechanism involved a PKA-dependent induction of a cyclin-dependent kinase inhibitor known as p27^{kip-1} that leads to growth arrest at the G1 phase of the cell cycle (Xaus et al. 1999). This molecular mechanism could potentially apply to the negative regulation of monocytic proliferation caused with elevated cAMP, regardless of the source, but this has not been fully tested in all cell systems. Using A_{2B} knockout mice and pharmacological inhibition of adenosine receptors several groups demonstrated that adenosine or ATP signaling through this receptor inhibits TLR-dependent IL-12p70 and TNF- α production in bone marrow derived DCs while enhancing IL-10 production (Addi Abduelhakem et al. 2008; Wilson et al. 2009). These cells were also less effective at stimulating T-cell proliferation, likely due to reduced MHC II and CD86 levels.

Studies were also performed in a mouse myeloid cell line to demonstrate the effects of cAMP on differentiation. Increasing cAMP levels using either 8-Br-cAMP or PGE₁ can induce macrophage differentiation in M1 myeloid cells, which were engineered to express the M-CSF receptor (Wilson et al. 2005). Increased cAMP can potentiate M-CSF-induced differentiation in cells containing the receptor through ERK activation, as differentiation was halted with the addition of a MEK inhibitor. These cells also retain the ability to inhibit M-CSF stimulated macrophage proliferation through phosphorylation of the M-CSF receptor. It has also been shown that cAMP can modulate M-CSF-induced MAPK activation of ERK, JNK, and p38 in a time-dependent manner, thereby inhibiting macrophage development (Zhu et al. 2008).

6 Conclusions

PDEs are a class of enzymes with a number of isoforms distributed throughout all tissues and cell types in the body. PDE5 inhibition has emerged as a proven method to treat erectile dysfunction and pulmonary hypertension. The ability to selectively inhibit a PDE in order to modulate cellular function is an exciting prospect for many future therapies. However, the administration of these inhibitors may carry unintended consequences when they affect the differentiation or activation of cells also containing the targeted PDE. For example, chronic administration of a PDE inhibitor would presumably increase the cyclic nucleotide levels in all cell types containing the targeted PDE. Observations of these increased levels are difficult to confirm *in vivo* and *in vitro* work utilizing monocyte or bone marrow-derived cells has been widely used as a surrogate for *in vivo* differentiation conditions.

Elevated cyclic nucleotide levels can have profound effects on the final phenotype of the cell when present during differentiation or maturation. Dendritic cell differentiation can be arrested in the presence of increased cAMP or cGMP at an intermediate stage between monocyte and dendritic cell. The capabilities of these dendritic cells are also altered, skewing its T-cell activation capacity toward a Th2 type response, largely due to the lack of IL-12 produced. Macrophage differentiation does not seem to be halted by elevated cyclic nucleotides, but a number of characteristics are altered. cAMP normally has anti-inflammatory actions on macrophages, but recent work has uncovered the role of cAMP in increasing chemokine and cytokine expression.

The alteration of hematopoietic differentiation by cyclic nucleotides and PDE inhibitors is also important regarding clinical efforts to generate therapeutic cell types from monocytes *ex vivo*. Generation of specific cell types from pluripotent and multipotent cells *ex vivo* for therapeutic usage holds great promise. For example, numerous efforts have been undertaken to develop DC or macrophage populations *ex vivo* that target specific antigens for fighting cancer (Paczesny et al. 2003), to treat chest wound infections after open heart surgery (Orenstein et al. 2005), to treat other refractory wounds (Zuloff-Shani et al. 2004), as a treatment for

spinal cord injury (Knoller et al. 2005), and as gene therapy delivery vehicles (Burke et al. 2002; Pastorino et al. 2001).

Although the phenotypes of the macrophages used for some therapeutic applications have been partly characterized, it is unclear which features are critical for their therapeutic effect. Currently, macrophages and other hematopoietic-derived cells are being generated *ex vivo* using serum and other mixed indeterminate stimuli. Using specific combinations of cytokines and humoral factors may allow generation of a more defined cell type with specific features that make it more therapeutically effective.

References

- Addi Abduehakeem B, Lefort A, Hua X, Libert F, Communi D, Ledent C, Macours P, Tilley SL, Boeynaems J-M, Robaye B (2008) Modulation of murine dendritic cell function by adenosine nucleotides and adenosine: involvement of the A2B receptor. *Eur J Immunol* 38:1610–1620
- Baillie GS, MacKenzie SJ, McPhee I, Houslay MD (2000) Sub-family selective actions in the ability of Erk2 MAP kinase to phosphorylate and regulate the activity of PDE4 cyclic AMP-specific phosphodiesterases. *Br J Pharmacol* 131:811–819
- Bang B, Ericson C, Aarbakke J (1994) Effects of cAMP and cGMP elevating agents on HL-60 cell differentiation. *Pharmacol Toxicol* 75:108–112
- Bar-Shavit Z (2007) The osteoclast: a multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. *J Cell Biochem* 102:1130–1139
- Bender AT, Beavo JA (2004) Specific localized expression of cGMP PDEs in Purkinje neurons and macrophages. *Neurochem Int* 45:853–857
- Bender AT, Beavo JA (2006a) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Bender AT, Beavo JA (2006b) PDE1B2 regulates cGMP and a subset of the phenotypic characteristics acquired upon macrophage differentiation from a monocyte. *Proc Natl Acad Sci USA* 103:460–465
- Bender AT, Ostenson CL, Giordano D, Beavo JA (2004) Differentiation of human monocytes *in vitro* with granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor produces distinct changes in cGMP phosphodiesterase expression. *Cell Signal* 16:365–374
- Bender AT, Ostenson CL, Wang EH, Beavo JA (2005) Selective up-regulation of PDE1B2 upon monocyte-to-macrophage differentiation. *Proc Natl Acad Sci USA* 102:497–502
- Boeynaems J-M, Communi D, Savi P, Herbert J-M (2000) P2Y receptors: in the middle of the road. *Trends Pharmacol Sci* 21:1–3
- Bornfeldt KE (2006) A single second messenger: several possible cellular responses depending on distinct subcellular pools. *Circ Res* 99:790–792
- Boss G (1989) cGMP-induced differentiation of the promyelocytic cell line HL-60. *Proc Natl Acad Sci USA* 86:7174–7178
- Brodsky A, Davio C, Shayo C, Lemos Legnazzi B, Barbosa M, Lardo M, Morelli A, Baldi A, Sanchez Avalos JC, Rivera E (1998) Forskolin induces U937 cell line differentiation as a result of a sustained cAMP elevation. *Eur J Pharmacol* 350:121–127
- Bryn T, Mahic M, Enserink JM, Schwede F, Aandahl EM, Tasken K (2006) The cyclic AMP-Epac1-Rap1 pathway is dissociated from regulation of effector functions in monocytes but acquires immunoregulatory function in mature macrophages. *J Immunol* 176:7361–7370
- Burke B, Sumner S, Maitland N, Lewis CE (2002) Macrophages in gene therapy: cellular delivery vehicles and *in vivo* targets. *J Leukoc Biol* 72:417–428

- Chaplinski TJ, Nield JE (1982) Cyclic nucleotide-induced maturation of human promyelocytic leukemia cells. *J Clin Invest* 70:953–964
- Cho Y-J, Kim J-Y, Jeong S-W, Lee SB, Kim ON (2003) Cyclic AMP induces activation of extracellular signal-regulated kinases in HL-60 cells: role in cAMP-induced differentiation. *Leuk Res* 27:51–56
- Cho E, Yu J, Kim M, Yim M (2004) Rolipram, a phosphodiesterase 4 inhibitor, stimulates osteoclast formation by inducing TRANCE expression in mouse calvarial cells. *Arch Pharm Res* 27:1258–1262
- Conti M, Richter W, Mehats C, Livera G, Park J-Y, Jin C (2003) Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J Biol Chem* 278:5493–5496
- Essayan DM (2001) Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol* 108:671–680
- Fernandez N, Monczor F, Lemos B, Notcovich C, Baldi A, Davio C, Shayo C (2002) Reduction of G protein-coupled receptor kinase 2 expression in U-937 cells attenuates H2 histamine receptor desensitization and induces cell maturation. *Mol Pharmacol* 62:1506–1514
- Fidock M, Miller M, Lanfear J (2002) Isolation and differential tissue distribution of two human cDNAs encoding PDE1 splice variants. *Cell Signal* 14:53–60
- Fischmeister R, Castro LRV, Abi-Gerges A, Rochais F, Jurevicius J, Leroy J, Vandecasteele G (2006) Compartmentation of cyclic nucleotide signaling in the heart: the role of cyclic nucleotide phosphodiesterases. *Circ Res* 99:816–828
- Fujita D, Yamashita N, Iita S, Amano H, Yamada S, Sakamoto K (2003) Prostaglandin E2 induced the differentiation of osteoclasts in mouse osteoblast-depleted bone marrow cells. *Prostaglandins Leukot Essent Fatty Acids* 68:351–358
- Gantner F, Kupferschmidt R, Schudt C, Wendel A, Hatzelmann A (1997a) In vitro differentiation of human monocytes to macrophages: change of PDE profile and its relationship to suppression of tumour necrosis factor-[alpha] release by PDE inhibitors. *Br J Pharmacol* 121:221–231
- Gantner F, Tenor H, Gekeler V, Schudt C, Wendel A, Hatzelmann A (1997b) Phosphodiesterase profiles of highly purified human peripheral blood leukocyte populations from normal and atopic individuals: a comparative study. *J Allergy Clin Immunol* 100:527–535
- Gantner F, Schudt C, Wendel A, Hatzelmann A (1999) Characterization of the phosphodiesterase (PDE) pattern of in vitro-generated human dendritic cells (DC) and the influence of PDE inhibitors on DC function. *Pulm Pharmacol Ther* 12:377–386
- Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K (2010) Development of monocytes, macrophages, and dendritic cells. *Science* 327:656–661
- Gilchrist M, Thorsson V, Li B, Rust AG, Korb M, Kennedy K, Hai T, Bolouri H, Aderem A (2006) Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* 441:173–178
- Giordano D, Magaletti DM, Clark EA, Beavo JA (2003) Cyclic nucleotides promote monocyte differentiation toward a DC-SIGN+ (CD209) intermediate cell and impair differentiation into dendritic cells. *J Immunol* 171:6421–6430
- Grage-Griebenow E, Flad HD, Ernst M (2001) Heterogeneity of human peripheral blood monocyte subsets. *J Leukoc Biol* 69:11–20
- Harris P, Ralph P (1985) Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. *J Leukoc Biol* 37:407–422
- Haskó G, Csóka B, Németh ZH, Vizi ES, Pacher P (2009) A2B adenosine receptors in immunity and inflammation. *Trends Immunol* 30:263–270
- Hertz AL, Bender AT, Smith KC, Gilchrist M, Amieux PS, Aderem A, Beavo JA (2009a) Elevated cyclic AMP and PDE4 inhibition induce chemokine expression in human monocyte-derived macrophages. *Proc Natl Acad Sci USA*. doi:10.1073/pnas.0911684106
- Hertz AL, Bender AT, Smith KC, Gilchrist M, Amieux PS, Aderem A, Beavo JA (2009b) Elevated cyclic AMP and PDE4 inhibition induce chemokine expression in human monocyte-derived macrophages. *Proc Natl Acad Sci* 106:21978–21983

- Heystek HC, Thierry A-C, Soulard P, Moulon C (2003) Phosphodiesterase 4 inhibitors reduce human dendritic cell inflammatory cytokine production and Th1-polarizing capacity. *Int Immunol* 15:827–835
- Holliday LS, Dean AD, Lin RH, Greenwald JE, Gluck SL (1997) Low NO concentrations inhibit osteoclast formation in mouse marrow cultures by cGMP-dependent mechanism. *Am J Physiol Renal Physiol* 272:F283–F291
- Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 35:91–100
- Houslay MD, Schafer P, Zhang KYJ (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10:1503–1519
- Itonaga I, Sabokbar A, Neale SD, Athanasou NA (1999) 1, 25-Dihydroxyvitamin D₃ and prostaglandin E₂ act directly on circulating human osteoclast precursors. *Biochem Biophys Res Comm* 264:590–595
- Jiang L, Foster FM, Ward P, Tasevski V, Luttrell BM, Conigrave AD (1997) Extracellular ATP triggers cyclic AMP-dependent differentiation of HL-60 cells. *Biochem Biophys Res Comm* 232:626–630
- Jonuleit H, Kühn U, Müller G, Steinbrink K, Paragnik L, Schmitt E, Knop J, Enk A (1997) Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol* 27:3135–3142
- Kalinski P, Hilkens CM, Snijders A, Snijdewint FG, Kapsenberg ML (1997) IL-12-deficient dendritic cells, generated in the presence of prostaglandin E₂, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* 159:28–35
- Kalinski P, Schuitmaker JHN, Hilkens CMU, Kapsenberg ML (1998) Prostaglandin E₂ induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. *J Immunol* 161:2804–2809
- Kinoshita T, Kobayashi S, Ebara S, Yoshimura Y, Horiuchi H, Tsutsumimoto T, Wakabayashi S, Takaoka K (2000) Phosphodiesterase inhibitors, pentoxifylline and rolipram, increase bone mass mainly by promoting bone formation in normal mice. *Bone* 27:811–817
- Knoller N, Auerbach G, Fulga V, Zelig G, Attias J, Bakimer R, Marder JB, Yoles E, Belkin M, Schwartz M, Hadani M (2005) Clinical experience using incubated autologous macrophages as a treatment for complete spinal cord injury: phase I study results. *J Neurosurg Spine* 3:173–181
- Kobayashi Y, Mizoguchi T, Take I, Kurihara S, Udagawa N, Takahashi N (2005a) Prostaglandin E₂ enhances osteoclastic differentiation of precursor cells through protein kinase A-dependent phosphorylation of TAK1. *J Biol Chem* 280:11395–11403
- Kobayashi Y, Take I, Yamashita T, Mizoguchi T, Ninomiya T, Hattori T, Kurihara S, Ozawa H, Udagawa N, Takahashi N (2005b) Prostaglandin E₂ receptors EP2 and EP4 are down-regulated during differentiation of Mouse osteoclasts from their precursors. *J Biol Chem* 280:24035–24042
- Kurland JI, Hadden JW, Moore MAS (1977) Role of cyclic nucleotides in the proliferation of committed granulocyte-macrophage progenitor cells. *Cancer Res* 37:4534–4538
- Kurland J, Bockman R, Broxmeyer H, Moore M (1978a) Limitation of excessive myelopoiesis by the intrinsic modulation of macrophage-derived prostaglandin E. *Science* 199:552
- Kurland J, Broxmeyer H, Pelus M, Bockman R, Moore M (1978b) Role for monocyte-macrophage-derived colony stimulating factor and prostaglandin E in the positive and negative feedback control of myeloid stem cell proliferation. *Blood* 52:388
- Legnazzi BL, Shayo C, Monczor F, Martin ME, Fernandez N, Brodsky A, Baldi A, Davio C (2000) Rapid desensitization and slow recovery of the cyclic AMP response mediated by histamine H₂ receptors in the U937 cell line. *Biochem Pharmacol* 60:159–166
- Li X, Okada Y, Pilbeam CC, Lorenzo JA, Kennedy CRJ, Breyer RM, Raisz LG (2000) Knockout of the murine prostaglandin EP2 receptor impairs osteoclastogenesis in vitro. *Endocrinology* 141:2054–2061

- Luft T, Jefford M, Luetjens P, Toy T, Hochrein H, Masterman K-A, Maliszewski C, Shortman K, Cebon J, Maraskovsky E (2002) Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E2 regulates the migratory capacity of specific DC subsets. *Blood* 100:1362–1372
- Mano M, Arakawa T, Mano H, Nakagawa M, Kaneda T, Kaneko H, Yamada T, Miyata K, Kiyomura H, Kumegawa M, Hakeda Y (2000) Prostaglandin E2 directly inhibits bone-resorbing activity of isolated mature osteoclasts mainly through the EP4 receptor. *Calcif Tissue Int* 67:85–92
- Miyamoto K-i, Waki Y, Horita T, Kasugai S, Ohya K (1997) Reduction of bone loss by denbufylline, an inhibitor of phosphodiesterase 4. *Biochem Pharmacol* 54:613–617
- Miyamoto K-i, Nishioka T, Waki Y, Nomura M, Katsuta H, Yokogawa K, Amano H (2006) Phosphodiesterase 4 inhibitor rolipram potentiates the inhibitory effect of calcitonin on osteoclastogenesis. *J Bone Miner Metab* 24:260–265
- Monczor F, Fernandez N, Riveiro E, Mladovan A, Baldi A, Shayo C, Davio C (2006) Histamine H2 receptor overexpression induces U937 cell differentiation despite triggered mechanisms to attenuate cAMP signalling. *Biochem Pharmacol* 71:1219–1228
- Morelli AE, Thomson AW (2003) Dendritic cells under the spell of prostaglandins. *Trends Immunol* 24:108–111
- Morita R, Ukyo N, Furuya M, Uchiyama T, Hori T (2003) Atrial natriuretic peptide polarizes human dendritic cells toward a Th2-promoting phenotype through its receptor guanylyl cyclase-coupled receptor A. *J Immunol* 170:5869–5875
- Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958–969
- Nakashima T, Takayanagi H (2009) Osteoclasts and the immune system. *J Bone Miner Metab* 27:519–529
- Noh ALSM, Yang M, Lee J-M, Park H, Lee D-S, Yim M (2009) Phosphodiesterase 3 and 4 negatively regulate receptor activator of nuclear factor-kappa B ligand-mediated osteoclast formation by prostaglandin E2. *Biol Pharm Bull* 32:1844–1848
- Nonaka T, Mio M, Doi M, Tasaka K (1992) Histamine-induced differentiation of HL-60 cells: the role of cAMP and protein kinase A. *Biochem Pharmacol* 44:1115–1121
- Novitskiy SV, Ryzhov S, Zaynagetdinov R, Goldstein AE, Huang Y, Tikhomirov OY, Blackburn MR, Biaggioni I, Carbone DP, Feoktistov I, Dikov MM (2008) Adenosine receptors in regulation of dendritic cell differentiation and function. *Blood* 112:1822–1831
- Olsson IL, Breitman TR, Gallo RC (1982) Priming of human myeloid leukemic cell lines HL-60 and U-937 with retinoic acid for differentiation effects of cyclic adenosine 3':5'-monophosphate-inducing agents and a T-lymphocyte-derived differentiation factor. *Cancer Res* 42:3928–3933
- Ono K, Akatsu T, Murakami T, Nishikawa M, Yamamoto M, Kugai N, Motoyoshi K, Nagata N (1998) Important role of EP4, a subtype of prostaglandin (PG) E receptor, in osteoclast-like cell formation from mouse bone marrow cells induced by PGE2. *J Endocrinol* 158:R1–R5
- Ono K, Kaneko H, Choudhary S, Pilbeam CC, Lorenzo JA, Akatsu T, Kugai N, Raisz LG (2005) Biphasic effect of prostaglandin E2 on osteoclast formation in spleen cell cultures: role of the EP2 receptor. *J Bone Miner Res* 20:23–29
- Orenstein A, Kachel E, Zuloff-Shani A, Paz Y, Sarig O, Haik J, Smolinsky AK, Mohr R, Shinar E, Danon D (2005) Treatment of deep sternal wound infections post-open heart surgery by application of activated macrophage suspension. *Wound Repair Regen* 13:237–242
- Paczynski S, Ueno H, Fay J, Banchereau J, Palucka AK (2003) Dendritic cells as vectors for immunotherapy of cancer. *Semin Cancer Biol* 13:439–447
- Park H, Yim M (2007) Rolipram, a phosphodiesterase 4 inhibitor, suppresses PGE2-induced osteoclast formation by lowering osteoclast progenitor cell viability. *Arch Pharm Res* 30:486–492
- Park H, Young Lee S, Lee D-S, Yim M (2007) Phosphodiesterase 4 inhibitor regulates the TRANCE/OPG ratio via COX-2 expression in a manner similar to PTH in osteoblasts. *Biochem Biophys Res Comm* 354:178–183

- Pastorino S, Massazza S, Cilli M, Varesio L, Bosco MC (2001) Generation of high-titer retroviral vector-producing macrophages as vehicles for in vivo gene transfer. *Gene Ther* 8:431–441
- Poppe H, Rybalkin SD, Rehmann H, Hinds TR, Tang X-B, Christensen AE, Schwede F, Genieser H-G, Bos JL, Doskeland SO, Beavo JA, Butt E (2008) Cyclic nucleotide analogs as probes of signaling pathways. *Nat Meth* 5:277–278
- Ransjö M, Lie A, Mackie EJ (1999) Cholera toxin and forskolin stimulate formation of osteoclast-like cells in mouse marrow cultures and cultured mouse calvarial bones. *Eur J Oral Sci* 107:45–54
- Reneland R, Mah S, Kammerer S, Hoyal C, Marnellos G, Wilson S, Sambrook P, Spector T, Nelson M, Braun A (2005) Association between a variation in the phosphodiesterase 4D gene and bone mineral density. *BMC Med Genet* 6:9
- Scandella E, Men Y, Gillessen S, Forster R, Groettrup M (2002) Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood* 100:1354–1361
- Shayo C, Davio C, Brodsky A, Mladovan AG, Legnazzi BL, Rivera E, Baldi A (1997) Histamine modulates the expression of c-fos through cyclic AMP production via the H2 receptor in the human promonocytic cell line U937. *Mol Pharmacol* 51:983–990
- Shayo C, Fernandez N, Legnazzi BL, Monczor F, Mladovan A, Baldi A, Davio C (2001) Histamine H2 receptor desensitization: involvement of a select array of G protein-coupled receptor kinases. *Mol Pharmacol* 60:1049–1056
- Shayo C, Legnazzi BL, Monczor F, Fernández N, Riveiro ME, Baldi A, Davio C (2004) The time-course of cyclic AMP signaling is critical for leukemia U-937 cell differentiation. *Biochem Biophys Res Comm* 314:798–804
- Shepherd MC, Baillie GS, Stirling DI, Houslay MD (2004) Remodelling of the PDE4 cAMP phosphodiesterase isoform profile upon monocyte-macrophage differentiation of human U937 cells. *Br J Pharmacol* 142:339–351
- Spina D (2008) PDE4 inhibitors: current status. *Br J Pharmacol* 155:308–315
- Takami M, Cho ES, Lee SY, Kamijo R, Yim M (2005) Phosphodiesterase inhibitors stimulate osteoclast formation via TRANCE/RANKL expression in osteoblasts: possible involvement of ERK and p38 MAPK pathways. *FEBS Lett* 579:832–838
- Take I, Kobayashi Y, Yamamoto Y, Tsuboi H, Ochi T, Uematsu S, Okafuji N, Kurihara S, Udagawa N, Takahashi N (2005) Prostaglandin E2 strongly inhibits human osteoclast formation. *Endocrinology* 146:5204–5214
- Tintut Y, Parhami F, Tsingotjidou A, Tetradis S, Territo M, Demer LL (2002) 8-Isoprostaglandin E2 enhances receptor-activated NFκB ligand (RANKL)-dependent osteoclastic potential of marrow hematopoietic precursors via the cAMP pathway. *J Biol Chem* 277:14221–14226
- Torphy TJ, Zhou HL, Cieslinski LB (1992) Stimulation of beta adrenoceptors in a human monocyte cell line (U937) up-regulates cyclic AMP-specific phosphodiesterase activity. *J Pharmacol Exp Ther* 263:1195–1205
- Tortora G, Clair T, Cho-Chung YS (1990) An antisense oligodeoxynucleotide targeted against the type II beta regulatory subunit mRNA of protein kinase inhibits cAMP-induced differentiation in HL-60 leukemia cells without affecting phorbol ester effects. *Proc Natl Acad Sci USA* 87:705–708
- Tortora G, Yokozaki H, Pepe S, Clair T, Cho-Chung YS (1991) Differentiation of HL-60 leukemia by type I regulatory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 88:2011–2015
- Tudhope SJ, Finney-Hayward TK, Nicholson AG, Mayer RJ, Barnette MS, Barnes PJ, Donnelly LE (2008) Different mitogen-activated protein kinase-dependent cytokine responses in cells of the monocyte lineage. *J Pharmacol Exp Ther* 324:306–312
- Vairo G, Argyriou S, Bordun AM, Whitty G, Hamilton JA (1990) Inhibition of the signaling pathways for macrophage proliferation by cyclic AMP. Lack of effect on early responses to colony stimulating factor-1. *J Biol Chem* 265:2692–2701

- Vergheze MW, McConnell RT, Lenhard JM, Hamacher L, Jin SL (1995) Regulation of distinct cyclic AMP-specific phosphodiesterase (phosphodiesterase type 4) isozymes in human monocytic cells. *Mol Pharmacol* 47:1164–1171
- Waki Y, Horita T, Miyamoto K-i, Ohya K, Kasugai S (1999) Effects of XT-44, a phosphodiesterase 4 inhibitor, in osteoblastogenesis and osteoclastogenesis in culture and its therapeutic effects. *Jpn J Pharmacol* 79:477–483
- Wani MR, Fuller K, Kim NS, Choi Y, Chambers T (1999) Prostaglandin E2 cooperates with TRANCE in osteoclast induction from hemopoietic precursors: synergistic activation of differentiation, cell spreading, and fusion. *Endocrinology* 140:1927–1935
- Wilson NJ, Cross M, Nguyen T, Hamilton JA (2005) cAMP inhibits CSF-1-stimulated tyrosine phosphorylation but augments CSF-1R-mediated macrophage differentiation and ERK activation. *FEBS J* 272:4141–4152
- Wilson JM, Ross WG, Agbai ON, Frazier R, Figler RA, Rieger J, Linden J, Ernst PB (2009) The A2B adenosine receptor impairs the maturation and immunogenicity of dendritic cells. *J Immunol* 182:4616–4623
- Xaus J, Valledor AF, Cardo M, Marques L, Beleta J, Palacios JM, Celada A (1999) Adenosine inhibits macrophage colony-stimulating factor-dependent proliferation of macrophages through the induction of p27kip-1 expression. *J Immunol* 163:4140–4149
- Yamagami H, Nishioka T, Ochiai E, Fukushima K, Nomura M, Kasugai S, Moritani S, Yokogawa K, Miyamoto K-i (2003) Inhibition of osteoclastogenesis by a phosphodiesterase 4 inhibitor XT-611 through synergistic action with endogenous prostaglandin E2. *Biochem Pharmacol* 66:801–807
- Zhu N, Cui J, Qiao C, Li Y, Ma Y, Zhang J, Shen B (2008) cAMP modulates macrophage development by suppressing M-CSF-induced MAPKs activation. *Cell Mol Immunol* 5:153–157
- Zuloff-Shani A, Kachel E, Frenkel O, Orenstein A, Shinar E, Danon D (2004) Macrophage suspensions prepared from a blood unit for treatment of refractory human ulcers. *Transfus Apher Sci* 30:163–167

Phosphodiesterase Inhibitors in the Treatment of Inflammatory Diseases

C.P. Page and D. Spina

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Abstract Phosphodiesterase 4 (PDE4) belongs to a family of enzymes which catalyzes the breakdown of 3, 5'-adenosine cyclic monophosphate (cAMP) and is ubiquitously expressed in inflammatory cells. There is little evidence that inflammatory diseases are caused by increased expression of this isoenzyme, although human inflammatory cell activity can be suppressed by selective PDE4 inhibitors. Consequently, there is intense interest in the development of selective PDE4 inhibitors for the treatment of a range of inflammatory diseases, including asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease, and psoriasis. Recent clinical trials with roflumilast in COPD have confirmed the therapeutic potential of targeting PDE4 and recently roflumilast has been approved for marketing in Europe and the USA, although side effects such as gastrointestinal disturbances, particularly nausea and emesis as well as headache and weight loss, may limit the use of this drug class, at least when administered by the oral route. However, a number of strategies are currently being pursued in attempts to improve clinical efficacy and reduce side effects of PDE4 inhibitors, including delivery via

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the inhaled route, development of nonemetic PDE4 inhibitors, mixed PDE inhibitors, and/or antisense biologicals targeted toward PDE4.

Keywords Asthma · Cilomilast · COPD · Emesis · Inflammation · PDE4 · Roflumilast

Abbreviations

COPD Chronic obstructive pulmonary disease

PDE Phosphodiesterase

1 Introduction

Theophylline has been used in the treatment of asthma and chronic obstructive pulmonary disease (COPD) since the 1930s, although its popularity has declined due to the introduction of β_2 -adrenoceptor agonists and glucocorticosteroids. Several mechanisms have been proposed to explain the potential beneficial action of theophylline in respiratory disease. Theophylline has been shown to inhibit the activity of a cyclic 3', 5' nucleotide PDE with a K_i of 100 μ M (Butcher and Sutherland 1962), which has been suggested to contribute to its ability to promote suppressor cell activity in lymphocytes (Shore et al. 1978; Zocchi et al. 1985; O'Shaughnessy et al. 2007) and its beneficial actions in patients with asthma (Sullivan et al. 1994), COPD (Rennard 2004), or psoriasis (Papakostantinou et al. 2005). However, theophylline has been relegated to second- or third-line therapy for the treatment of respiratory disease and is not routinely used in the treatment of other inflammatory conditions such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease, in part because it is not recognized as an anti-inflammatory drug, but more importantly because of the very significant drug interactions and narrow therapeutic window exhibited by theophylline; the side effect profile of theophylline includes nausea, emesis, gastrointestinal disturbances, and arrhythmias. These problems, in part, promoted an interest in understanding how the PDE family of enzymes regulated cAMP levels within cells and the potential for developing drugs inhibiting such enzymes, in a selective way as a way of improving the therapeutic window of theophylline.

There are presently 11 known families of PDEs and at least 21 isoforms with numerous splice variants that are characterized by differences in structure, substrate specificity, inhibitor selectivity, tissue and cell distribution, regulation by kinases, protein-protein interaction, and subcellular distribution (Conti and Beavo 2007; McCahill et al. 2008). However, targeting PDE4, an enzyme family that exclusively metabolizes cAMP (Houslay et al. 2005, 2007), has been the focus for the development of drugs that could prove beneficial in the treatment of depression, schizophrenia (Millar et al. 2007), respiratory diseases such as asthma and COPD

(Barnes 2008), allergic skin diseases and psoriasis (Baumer et al. 2007), and inflammatory bowel disease (Keshavarzian et al. 2007).

It is therefore of interest that plasma levels achieved with a dose of theophylline that demonstrated significant anti-inflammatory activity (Sullivan et al. 1994) are well below the K_i for PDE inhibition and suggested that PDE4 inhibition alone does not completely explain the clinical effectiveness of this drug (Barnes et al. 2005). Nevertheless, highly potent and selective PDE4 inhibitors have been developed to target a range of inflammatory diseases. However, targeting PDE enzymes is not unique to inflammatory diseases as exemplified with the development and clinical success of a number of PDE5 inhibitors for the treatment of erectile dysfunction as exemplified by sildenafil (Boolell et al. 1996).

2 Phosphodiesterase 4

PDE4 is highly selective for catalyzing the hydrolysis of cAMP, which terminates the downstream signalling of this second messenger (Houslay et al. 2007; Houslay 2010). There are 4 gene families (A, B, C, and D), where alternative mRNA splicing and the use of distinct promoters generates more than 20 splice variants (Houslay et al. 2007). While hydrolysis of cAMP is a common feature of this family, it is clear that these isoforms can be targeted to different domains and signalling complexes within the intracellular compartment, where they play a pivotal role in underpinning compartmentalized cAMP signaling (Houslay 2010). Furthermore, their activities can be differentially regulated by phosphorylation by various protein kinases, suggesting that these isoforms have specific functions in the control of cellular activity (McCahill et al. 2008).

X-ray crystallography has resolved the catalytic domain of these enzymes, which comprise three important domains: a bivalent metal binding pocket (Zn^{2+} , Mg^{2+}), which is thought to form a complex with the phosphate moiety of cAMP, a pocket containing an invariant glutamine (Q pocket), which forms hydrogen bonds with the nucleotide (purine) moiety of cAMP, and a solvent pocket. PDE4 inhibitors occupy this active site through a number of important interactions and prevent cAMP metabolism. These include indirect binding to the metal ions via the formation of hydrogen bonding to water while hydrophobic interactions between the planar ring structure of these inhibitors and hydrophobic amino acid residues such as phenylalanine and isoleucine serve to “clamp” the inhibitor within the active site. There are also hydrogen bond interactions between the aromatic ring structure of these inhibitors and the invariant glutamine residue in the Q pocket, the site which is normally occupied by the nucleotide moiety of cAMP (Xu et al. 2000; Card et al. 2004; Wang et al. 2007).

There are considerable challenges to the synthesis of subtype selective inhibitors due to the high degree of sequence and structural homology within the catalytic domains of the PDE4 subtypes (Xu et al. 2000; Card et al. 2004;

Houslay et al. 2005; Wang et al. 2007). One possibility might be to exploit subtle differences between the interaction of these inhibitors with the catalytic active site. Alternatively, compounds that inhibit catalysis by targeting regions outside the catalytic site may be possible. Compounds that selectively interact with the N terminal regions of the respective PDE4, which contain phosphorylation sites and/or protein binding sequences, might indirectly interfere with PDE4 activity (Houslay et al. 2005). Moreover, inhibitors may act through a combined interaction with residues in regions that fold over or near the catalytic site, e.g., UCR2 which provides a regulatory region unique to PDE4 enzymes as well as some residues in the catalytic domain (Kranz et al. 2009; Burgin et al. 2010).

PDE4 is expressed in a number of cell types that are considered important in the pathogenesis of inflammatory disease (Table 1). It might reasonably be argued that targeting the inhibition of PDE4 could potentially suppress the function of numerous cell types. However, it is well known that other PDE enzymes are also expressed in these cells and the contribution of other PDEs to cell function (e.g., PDE3, PDE7) in the context of regulating inflammatory cell function is also being explored (Smith et al. 2004; Boswell-Smith et al. 2006a; Banner and Press 2009). Although it would seem prudent to develop subtype selective PDE4 inhibitors in attempts to maximize therapeutic benefit at the expense of adverse effects, there is also the possibility that nonselective PDE inhibitors might offer a better approach in targeting multiple target cells in the disease process. Indeed, it has been suggested that clozapine is a better antipsychotic than newer generation atypical antipsychotics because this drug targets numerous receptors and as such, has been described as a “magic shotgun” for the treatment of schizophrenia (Roth et al. 2004).

3 Asthma and PDE4

The underlying pathophysiological features of asthma are a consequence of the contribution of numerous cells including the epithelium, dendritic cells, T-lymphocytes, eosinophils, mast cells, and airway smooth muscle. Thus, there is a complex web of interconnecting interactions between different cell types and inflammatory mediators. Whilst glucocorticosteroids remain the mainstay treatment of this disease, there still remains a group of asthmatics with poor symptom control despite treatment with these drugs (Barnes 2008). It is therefore of interest that many of the cell types illustrated in Table 1 express PDE4 and inhibition of this enzyme family can suppress the function of these cells.

A number of studies have investigated whether the pathogenesis of asthma might be a consequence of increased PDE4 protein expression and activity. This hypothesis had some currency in view of studies in the field of dermatology, which purported to demonstrate the expression of a novel and distinct monocyte-derived cAMP-PDE obtained from individuals with atopic dermatitis. Monocytes from these individuals had increased functionality and this corresponded with the presence of protein with increased PDE activity. This was thought to be

Table 1 PDE distribution within human cells of interest for the treatment of respiratory diseases such as asthma and COPD

Cell type	PDE4 subtype ^a	Other PDE's	Biological consequence of PDE4 inhibition	Reference
T-lymphocytes CD4 CD8 Th1, Th2 Th17	A, B, D ^b	3, 7	Inhibition of proliferation and cytokine release	Essayan et al. (1997), Gantner et al. (1997b), Hatzelmann and Schudt (2001), Smith et al. (2003), Peter et al. (2007), Ma et al. (2008)
B cells	A, B, D	7	Increased proliferation	Gantner et al. (1998), Smith et al. (2003)
Eosinophils	A, B, D	7	Inhibition of superoxide anion generation; delayed apoptosis	Hatzelmann and Schudt (2001), Smith et al. (2003), Parkkonen et al. (2007)
Neutrophils	A, B, D	7	Inhibition of superoxide anion and neutrophil elastase release	Hatzelmann and Schudt (2001), Smith et al. (2003), Jones et al. (2005)
Monocyte	A, B, D	7	Inhibition of TNF α release	Hatzelmann and Schudt (2001), Smith et al. (2003), Heystek et al. (2003), Jones et al. (2005)
Macrophages	A, B, D	1, 3, 7	Inhibition of TNF α release ^c	Gantner et al. (1997a), Hatzelmann and Schudt (2001), Smith et al. (2003), Barber et al. (2004)
Dendritic cells	A, B, D	1, 3	Inhibition of TNF α release	Hatzelmann and Schudt (2001), Heystek et al. (2003)
Osteoblast	A, B, D	3	Stimulates RANKL-induced osteoclast formation	Noh et al. (2009)
Chondrocytes	A, B, D	1	Inhibition of IL-1 β stimulated production of nitric oxide	Tenor et al. (2002)
Mast cells			Little if any mast cell stabilization	Weston et al. (1997), Shichijo et al. (1998)
Airway epithelial cells		1-3, 4, 5, 7, 8	Increased production of PGE ₂ ; inhibition of IL-6 production	Fuhrmann et al. (1999), Haddad et al. (2002)
Endothelial cells		2, 3, 4, 5	Inhibition of adhesion molecule expression	Jones et al. (2005), Sanz et al. (2007)
Fibroblasts	A, B, D	1, 4, 5, 7	Inhibition of fibroblast chemotaxis; inhibition of pro-MMP1,2 release Differentiation into myofibroblasts	Kohyama et al. (2002), Smith et al. (2003), Martin-Chouly et al. (2004), Dunkern et al. (2007)
^d Sensory nerves	D	1, 3	Inhibition of neuropeptide release	Spina et al. (1995)

^aPDE4 subtype mRNA expression illustrating relative abundance in cells^bPDE4D absent in Th1 cells^cIn the presence of a PDE3 inhibitor^dGuinea-pig sensory nerves

the underlying basis for the pathology associated with atopic dermatitis (Chan et al. 1993). However, soluble PDE4 activity was not increased in a range of peripheral blood leukocytes from atopic subjects of either mild or severe severity (Gantner et al. 1997b). Similarly, while increased total PDE catalytic activity was observed in peripheral blood monocytes from individuals with mild asthma, this was associated, paradoxically, with reduced PDE4 activity (Landells et al. 2001), and the expression of PDE4A, B, and D was not increased in peripheral blood CD4 positive T-lymphocytes in patients with mild asthma (Jones et al. 2007). Together these studies indicate that the underlying pathogenesis of mild asthma cannot be attributed to enhanced PDE4 expression or activity. A genome-wide association search has demonstrated single nucleotide polymorphisms on chromosome 5q12 for PDE4D (Himes et al. 2009).

The significance of these findings remains to be established given that this isoform is purportedly linked with emesis and targeting of PDE4A and PDE4B is seen as a rationale approach to develop nonemetic anti-inflammatory PDE4 inhibitors (Manning et al. 1999; Kranz et al. 2009). If this assertion is correct, then the challenge remains to develop a PDE4 subtype selective inhibitor that differs by at least three orders of magnitude, but this has yet to be achieved after more than a decade of work in this field. However, this hypothesis has been questioned following the discovery of PDE4D selective allosteric inhibitors, which promote anti-inflammatory activity and are largely devoid of emesis in animal models (Burgin et al. 2010). The implication of these novel findings is that selectivity for different PDE4 subtypes is not a critical determinant in avoiding side effects such as nausea, but whether partial inhibition can be achieved, as in this example using allosteric inhibitors which interact with upstream conserved regions (UCRs), and the catalytic domain of PDE4D (Burgin et al. 2010).

Numerous preclinical studies in models of allergic pulmonary inflammation have repeatedly documented the ability of PDE4 inhibitors to inhibit two important characteristic features of asthma, namely, the recruitment of eosinophils to the airways and bronchial hyperresponsiveness (Spina 2003). One disadvantage of these studies is the inability to ascertain the role of PDE4 isoforms because of the nonselective nature of the PDE4 inhibitors currently under development. However, the use of genetically modified mice has revealed some interesting findings. Airway inflammation characterized by recruitment of eosinophils to the airways of mice deficient in PDE4D was not different from that of wild-type controls (Hansen et al. 2000). This indicated that other PDE4 subtypes contribute in the metabolism of intracellular cAMP since cell recruitment to the airways was inhibited when animals were treated with nonselective PDE4 inhibitors (Kung et al. 2000; Kanehiro et al. 2001). However, airway obstruction caused by methacholine was enhanced in wild-type allergic mice, but abolished in PDE4D gene-deficient mice. These mice were *hyporesponsive* to methacholine, even in the absence of allergic sensitization, and this feature appeared to be related to an increase in the production of a dilator prostaglandin in the airways of these PDE4D gene-deficient mice (Hansen et al. 2000; Mehats et al. 2003). However, this effect was specific for methacholine because the enhanced airway obstruction in response to serotonin

was unaffected by the removal of PDE4D (Hansen et al. 2000). This highlights the potential complementary role of PDE4 isoforms in regulating allergic airway inflammation, and the need to target more than one PDE4 isoform since the inflammatory response, bronchial hyperresponsiveness, and airway remodeling in allergic wild-type mice could be inhibited by nonselective PDE4 inhibitors such as roflupram or roflumilast (Kung et al. 2000; Kanehiro et al. 2001; Kumar et al. 2003). Whilst the use of gene knockout studies can offer insights into the function of proteins, it is likely that compensatory mechanisms at birth and the mixed background of the generated knockouts could confound attempts to define the biological role of a particular protein. The use of conditional PDE4 knockouts or a biological approach (e.g., siRNA) may be required to understand the biological role(s) of PDE4 subtypes completely (Huston et al. 2008).

The numerous preclinical studies reporting the anti-inflammatory potential of PDE4 inhibitors in models of allergic inflammation and in human cells *in vitro* have to some degree been corroborated in clinical trials in asthmatic subjects. Twice-daily treatment for 9.5 days with the PDE4 inhibitor CDP840 inhibited the development of the late phase response in asthmatic subjects by 30% (Harbinson et al. 1997). A similar degree of inhibition of the late phase response was observed following once daily treatment for 7- to 10-day with roflumilast (van Schalkwyk et al. 2005), while 2-week treatment with MK-0359 improved baseline FEV1 and reduced symptom scores in chronic asthmatic subjects (Lu et al. 2009). This late phase response is used by clinicians to model the inflammatory component following an allergic insult to the airways. In both allergen challenge studies, the effect of drug treatment on the acute allergen bronchoconstriction was modest and consistent with the lack of demonstrable action of PDE4 inhibition on both mast cell and airway smooth muscle function. This highlights the role of other PDE enzymes, namely PDE3 in the regulation of the function of these cell types in the airway (Table 1). Bronchial hyperresponsiveness was not reduced by these drugs, with only one study purporting to show modest protection against allergen-induced bronchial hyperresponsiveness (Louw et al. 2007). This suggests that PDE4 may not be a suitable target for this particular phenomenon, or that higher doses are required to provide complementary and persistent inhibition of the enzyme and hence attenuation of bronchial hyperresponsiveness. It is of interest that roflumilast has a plasma half-life of 16 h following a single oral administration and is metabolized by CYP3A4 to the active N-oxide metabolite, which has considerably greater bioavailability with a half-life of 20 h that would favor prolonged enzyme exposure (David et al. 2004). This favorable pharmacokinetic profile would be anticipated to produce longer periods of PDE4 inhibition. However, whilst there was a significant reduction in the activity of circulating monocytes in subjects maintained on roflumilast for 4 weeks, the magnitude of this change was small, resulting in only an approximately 1.3-fold reduction in TNF α production by monocytes in response to endotoxin challenge *in vitro* (Timmer et al. 2002). One could argue that only a partial inhibition of PDE4 activity was achieved at the dose employed in clinical studies and consequently suppression of inflammatory cell function within the airway tissue compartment is not maximal, a hypothesis borne out in several clinical studies (Gamble et al. 2003; Grootendorst et al. 2007).

Unfortunately, the doses that can be administered clinically with most orally active PDE4 inhibitors are limited by side effects, the most commonly reported being headache, nausea, and diarrhea of a mild-to-moderate severity (van Schalkwyk et al. 2005; Lu et al. 2009). The availability of other nonemetic drugs such as CDP840 (Harbinson et al. 1997) might provide hope that such PDE4 inhibitors can be developed for the treatment of asthma, a worthy goal to pursue in light of at least one clinical study reporting comparable clinical efficacy between roflumilast and the inhaled glucocorticosteroids beclomethasone dipropionate in persistent asthma (Bousquet et al. 2006). Such systemic side effects may also be eliminated by using the inhaled route such that relatively higher doses of PDE4 inhibitor can be delivered to the intrapulmonary compartment whilst reducing systemic bioavailability as recently reported with GSK256066 (Singh et al. 2010). However, another inhaled PDE inhibitor, UK 50,000 failed to show any clinical benefit (Phillips et al. 2007) and an early mixed PDE 3/4 inhibitor zardaverine actively elicited frank emesis at inhaled doses that induced bronchodilation (Brunnee et al. 1992). Mesenteric arteritis, characterized by inflammation of the arterioles is observed in rodents and primates following doses of some PDE4 inhibitors far exceeding those that would be used clinically; however, this condition has not been observed in subjects in clinical trials with roflumilast and therefore is unlikely to be a major issue and even less so if inhaled formulations of this drug class are developed (Spina 2008).

4 COPD and PDE4

Unlike asthma, COPD is primarily caused by cigarette smoking, although in developing countries smoke derived from burning biomass fuels is also a predisposing factor (Salvi and Barnes 2009). COPD is also an inflammatory disease but the nature of the inflammatory response is distinct from asthma. The inflammatory response in COPD is characterized by the activation of macrophages and airway epithelial cells, which in turn, secrete a range of chemokines and lipid mediators resulting in the recruitment of neutrophils and CD8+ T-lymphocytes to the lung. The secretion of a range of proteases from neutrophils (elastase, MMP9, cathepsins) and macrophages (MMP12) is thought to contribute toward fibrosis of the small airways and increased mucus secretion and destruction of the alveolar wall (Barnes 2008) These pathological changes give rise to the symptoms of cough, mucus secretion, dyspnea, and emphysema. Many of the cell types implicated in the COPD inflammatory response express PDE4 (Table 1).

The expression of PDE4A-D mRNA transcripts in peripheral blood neutrophils and CD8 T cells is not altered in subjects with mild COPD (Jones et al. 2007). However, the expression of PDE4A4 mRNA transcript was significantly increased in macrophages purified from bronchoalveolar lavage fluid from subjects with mild-to-moderate COPD compared with healthy subjects or smokers who did not present with COPD (Barber et al. 2004). Of the 12 PDE4 variants analyzed, only the

activity of PDE4A4 was increased and suggested that local events/processes within the lung of subjects with COPD specifically upregulated this variant (Barber et al. 2004). However, the functional consequence of this change remains to be established in light of findings showing that PDE4 inhibition has only a modest effect in suppressing TNF α production from human macrophages derived from cultured monocytes. Moreover, the contribution of other PDEs (e.g., PDE3, PDE7) and other PDE4 isoforms in regulating function in this cell type clearly need to be explored (Gantner et al. 1997a; Smith et al. 2004). Genotyping studies have also revealed the presence of single nucleotide polymorphisms within the PDE4D gene in COPD subjects compared with smokers within the Japanese population that was in linkage disequilibrium with the IL-13 gene. However, the significance of these findings remains to be established since this relationship was absent in an Egyptian population (Homma et al. 2006).

In vivo models of COPD are very limited. However, the recruitment of neutrophils to the airways can be readily induced using the bacterial wall component, endotoxin, although it is widely appreciated that this stimulus can only model neutrophil recruitment to the airways (Leclerc et al. 2006). Neutrophil recruitment into the airways of wild-type mice was inhibited by around 50% in PDE4B and PDE4D-deficient mice and a greater degree of inhibition was observed when wild type mice were treated with rolipram (Ariga et al. 2004). This once again highlights the complementary roles of PDE4 isoforms in regulating neutrophil recruitment to the airways. Similarly, smoking-induced neutrophil recruitment to the airways, release of chemokines, and emphysematous changes to the lung were attenuated by PDE4 inhibitors (Martorana et al. 2005; Leclerc et al. 2006). Together, these studies highlight the utility of inhibiting PDE4 in cell types implicated in COPD.

A number of phase III clinical trials have assessed the potential utility of PDE4 inhibitors in the treatment of COPD (Rabe et al. 2005; Rennard et al. 2006; Calverley et al. 2007). All three studies report modest, but nevertheless significant improvements in spirometry over placebo, quality of life scores, and reduction in the number of exacerbations in the severest group of COPD subjects. Indeed, recent phase III clinical trials evaluating the effect of roflumilast in symptomatic moderate-to-severe asthmatics have reported more promising findings (Fabbri et al. 2009; Calverley et al. 2009). Roflumilast caused a significant 48-ml improvement in FEV1 compared with placebo and reduced exacerbation rates of moderate-to-severe intensity by 17% over a 52-week period (Calverley et al. 2009). Similarly, roflumilast consistently improved baseline FEV1 in moderate-to-severe COPD patients already taking either a long-acting β_2 -agonist (salmeterol) or a long-acting muscarinic antagonist (tiotropium bromide) by 49 and 80 ml, respectively, during 24 weeks of treatment (Fabbri et al. 2009).

The mechanism of the improvement in spirometry is unlikely to be due to relaxation of airway smooth muscle because this drug class has weak bronchodilator activity. It is possible that this improvement is due to an anti-inflammatory action of the drugs (Table 1), although no biomarker of inflammation was measured in these studies. However, separate studies have addressed whether PDE4

inhibitors are anti-inflammatory in patients with COPD. Both roflumilast (Grootendorst et al. 2007) and cilomilast (Gamble et al. 2003) reduced both the number of inflammatory cells, such as, neutrophils and lymphocytes recruited to the airways and the levels of two biochemical markers of this disease, namely IL-8 and neutrophil elastase. The magnitude of the change in the number of these inflammatory cells and concentration of mediators was between 30 and 50%, which might contribute to the beneficial action of these two PDE4 selective in the clinic trials. However, to date the substantial inhibition of PDE4 with an orally active inhibitor has not been achieved because of dose-limiting side effects, which may also explain the modest clinical benefit shown with these drugs. As discussed for asthma, it is plausible that other PDE species (e.g., PDE3, 7; see Table 1) may require targeting for a full anti-inflammatory therapeutic action to be attained in treating this disease (Banner and Press 2009).

The most common side effect reported with roflumilast included diarrhea (9%), headache (5%), and nausea (5%) (Rabe et al. 2005; Calverley et al. 2007, 2009; Fabbri et al. 2009). This was of the same order of magnitude as reported with cilomilast, although abdominal pain and vomiting were also reported for this drug (Rennard et al. 2006). The adverse effects seemed to disappear with continued use; nonetheless, these side effects were a major reason why patients did not continue with the study during the first 3–4 weeks of treatment. No cardiovascular liabilities were noted. One unanticipated side effect that was noted following prolonged treatment with roflumilast was a significant reduction in body weight in subjects. This might be attributed to gastrointestinal discomfort in susceptible patients, although it was interesting to note that weight loss was also reported in patients not complaining of gastrointestinal side effects and might be an effect worthy of further study (Fabbri et al. 2009; Calverley et al. 2009).

One might anticipate that the risk/benefit ratio would be improved by inhaled delivery of a PDE4 inhibitor directly to the lung. Unfortunately, to date only one study has evaluated the effect of an inhaled PDE4 nonselective subtype inhibitor. This was used to treat moderate-to-severe COPD and no significant improvement in baseline FEV1 was observed after 6 weeks of treatment (Vestbo et al. 2009). As no inflammatory biomarker was measured in this study, it is unclear whether a sufficient level of sustained PDE4 inhibition was achieved. This seems likely because adverse events such as headache and improvements in lung function that were noted at 2 weeks were absent after 6 weeks of treatment with the high dose. Whether inhaled PDE4 inhibitors will be of utility in COPD remains to be determined but there may be a cause for optimism in view of the amelioration of the allergen-induced late phase response in asthma with the inhaled PDE4 inhibitor GSK256066 (Singh et al. 2010). This demonstrates that in principle one can achieve inhibition of PDE4 following topical delivery to the lung that could lead to clinical benefit, but the relevant clinical trials need to be undertaken with GSK256066 to ascertain whether this drug will be of any clinical benefit to patients with COPD.

5 Other Inflammatory Diseases and PDE4

Numerous inflammatory conditions such as psoriasis (Nestle et al. 2009), rheumatoid arthritis (Taylor and Feldmann 2009), and inflammatory bowel disease (Abraham and Cho 2009) are characterized by a complex interplay between cells resulting in their activation and recruitment, release of proinflammatory cytokines, and tissue damage. Biologicals directed against cytokines are one of the most important therapies to emerge in the past decade and have revolutionized the treatment of inflammatory diseases as exemplified by those targeting TNF α (Nestle et al. 2009; Taylor and Feldmann 2009; Abraham and Cho 2009). It is therefore of interest that PDE4 inhibitors can suppress TNF α production by various inflammatory cell types, and inhibit the activity of immune cells so critical in these diseases (Table 1). It is therefore no surprise that PDE4 inhibitors are also being considered for the treatment of a number of nonrespiratory inflammatory diseases.

Earlier observations suggested the targeting of cAMP PDE might prove beneficial in the treatment of skin inflammatory disease. For example, potential deficits in adenylyl cyclase signaling in psoriasis (Wright et al. 1973) and expression of a novel monocyte-derived cAMP PDE in atopic dermatitis (Chan et al. 1993) were reported. Consequently, several selective PDE4 inhibitors have been evaluated in clinical trials. Patients with psoriasis and treated with apremilast, an orally active PDE4 inhibitor demonstrated an improvement in clinical scores and reduced T-cell number in the dermis in an open-label clinical trial (Gottlieb et al. 2008). Similarly, the systemic administration of apremilast reduced pathological features of psoriatic human skin transplanted onto immunocompromised mice (Schafer et al. 2010). One of the advantages of inflammatory dermatoses is that topical administration could limit the systemic liability associated with orally administered PDE4 inhibitors and this strategy has been adopted for the development of AN2728 for the treatment of psoriasis and atopic dermatitis with promising results in phase II clinical trials (Nazarian and Weinberg 2009; Akama et al. 2009).

Inflammatory bowel diseases might also be amenable to treatment with PDE4 inhibitors since Th1 and Th17 cells are implicated in this disease and the activity of these cell types can be suppressed by PDE4 inhibitors (Table 1). The PDE4 inhibitor, tetomilast, appeared to reduce various indices of inflammation in IL10-deficient mice that spontaneously develop chronic gastrointestinal inflammation characterized by mucus secretion, rectal prolapse, and diarrhea (Ichikawa et al. 2008). Tetomilast has also been evaluated in a phase II clinical trial in active ulcerative colitis (Schreiber et al. 2007). Improvement in disease as defined by a disease activity index of ≥ 3 was achieved in 53% (25 mg dose) and 39% (50 mg dose) of patients after 8 weeks of treatment, although this was not statistically different from the placebo group. However, post-hoc analysis revealed that patients with more severe disease showed significant improvement when treated with tetomilast. One of the potential issues with this study is the reporting of nausea and emesis, particularly with the higher dose of tetomilast which will be of concern when treating patients with gastrointestinal inflammation.

Rheumatoid arthritis is amenable to anti-TNF α monoclonal antibodies, which highlights an important role this cytokine plays in disease pathology. Similarly, T cells (e.g., Th1, Th17), macrophages, chondrocytes, and osteoblasts all play a role in this disease (Nestle et al. 2009). All these cell types express PDE4 and inhibition of this enzyme can lead to a reduction in cell activity that might be conducive to a beneficial action in this disease (Table 1).

Preclinical studies have shown that PDE4 inhibitors can suppress pannus-like inflammation in animals injected with methylated bovine serum albumin. Furthermore, PDE4 inhibitors suppressed cytokine production from peritoneal macrophages and inhibited synovial fibroblast proliferation in culture (Kobayashi et al. 2007). The recent finding that PDE4 inhibitors suppressed IL-17 production from human peripheral blood mononuclear cells and CD4+ T lymphocytes and inhibited proliferation of human memory Th17 (Ma et al. 2008) could explain the preclinical findings. Indeed, the expression of IL-23 from human peripheral blood mononuclear cells, a cytokine which supports the expansion of Th17 cells was suppressed by PDE4 inhibition (Schafer et al. 2010). Similarly, roflumilast partially attenuated nitric oxide release from human chondrocytes in culture, these cells are involved in articular cartilage destruction in rheumatoid arthritis (Tenor et al. 2002). Loss of bone is also a feature of rheumatoid arthritis, and PDE3 and PDE4 inhibitors have been shown to augment the ability of prostaglandin E2 to promote murine osteoclast differentiation in co-cultures of calvarial osteoclasts and bone marrow cells (Noh et al. 2009). However, it is well established that PGE2 promotes bone formation in vivo, and this anabolic effect is also observed with PDE4 inhibitors in ovariectomized rats (Yao et al. 2007). PDE4 inhibitors appear to suppress inflammation, cartilage destruction, and bone loss in rheumatoid arthritis, but it remains to be established whether this drug class will be significantly better than current biological agents.

6 Unwanted Side Effects

Nausea and other gastrointestinal side effects are commonly reported side effects associated with many PDE4 inhibitors and the mechanism responsible for these side effects has been investigated in an attempt to discover nonemetic PDE4 inhibitors. The direct recording of neuronal activity within the area postrema of dogs conclusively demonstrated that substances known to cause nausea (e.g., apomorphine) caused the excitation of neurons within this anatomical location (Carpenter et al. 1988). Neuronal activity within the area postrema was also increased following the systemic administration of 8-bromo cAMP or following elevation of endogenous levels of cAMP within neurones by forskolin, an activator of adenylyl cyclase (Carpenter et al. 1988). Elevated levels of cAMP within the area postrema enhanced the emetogenic response. Dogs treated with theophylline and the PDE4 selective inhibitor, 4-(3-Butoxy-4-methoxyphenyl)methyl-2-imidazolidone (Ro 20-1724)

reduced the emetic threshold of the D2 agonist, apomorphine (Carpenter et al. 1988). One consequence of elevated levels of cAMP is the transcriptional activation of the early response gene *c-fos*, following upstream activation of the transcription factor, cAMP response element binding (CREB) by protein kinase A. This method was employed to demonstrate increased *c-fos* immunoreactivity in neurons within area postrema and nucleus tractus solitarius following systemic administration of a PDE4 inhibitor and providing conclusive proof that this drug class can lead to the activation of neurons within the emetic centers of the central nervous system (CNS) (Bureau et al. 2006). Direct application of a highly potent PDE4 inhibitor via intracerebroventricular administration in order to limit systemic bioavailability provide a means of directly activating neurons within the CNS, consequently resulting in emesis in the ferret (Robichaud et al. 1999). This ability of PDE4 inhibitors to induce emesis in the ferret was inhibited by the α_2 -selective agonist, clonidine (Robichaud et al. 2001) and suggested that raising cAMP within central noradrenergic terminals by PDE4 inhibitors promoted emesis, and this could be attenuated via α_2 -adrenoceptor mediated inhibition of adenylyl cyclase.

The emetic response to systemically administered PDE4 inhibitors is reduced by anti-emetic agents including the 5HT₃-antagonist, ondansetron, and the NK₁ antagonist, (+)-(2*S*,3*S*)-3-(2-[¹¹C]Methoxybenzylamino)-2-phenylpiperidine (CP-99,994) (Robichaud et al. 1999, 2001). Similarly, the increased expression of *c-fos* within the emetic centers of the brain was also reduced following treatment with the NK₁ antagonist, RP67580, thus implicating substance P in this response (Bureau et al. 2006).

Many studies have documented the expression of PDE4D within the area postrema, nucleus tractus solitarius, and nodose ganglion neurons in various species including man and implicated this isoform in nausea and vomiting (Cherry and Davis 1999; Takahashi et al. 1999; Perez-Torres et al. 2000; Lamontagne et al. 2001). However, it should also be recognized that detectable transcripts for PDE4B were also found within the nucleus tractus solitarius and area postrema in humans and rodents, respectively, and could just as well be involved in the emetic response (Perez-Torres et al. 2000). Consequently, there is a general consensus that inhibition of PDE4D is responsible for side effects such as nausea and emesis. Since rodents lack an emetic reflex, it is not possible to directly investigate the role of different isoforms of PDE4 in emesis in this model. However, a surrogate biological response, which measures the reversal of anesthesia induced by α_2 -adrenoceptor agonists (e.g., clonidine, xylazine), has been used to study the role of PDE4 subtypes in emesis (Robichaud et al. 2001, 2002a). Deletion of PDE4D and not PDE4B reduced the duration of anesthesia induced by xylazine, compared with wild-type mice, and the ability of PDE4 inhibitors to shorten xylazine-induced anesthesia was impaired in PDE4D but not PDE4B knockout mice (Robichaud et al. 2002b). Together these studies suggested that PDE4 inhibitors with low affinity for PDE4D should have reduced emetic potential.

However, the validity of this hypothesis has been questioned in light of the development of PDE4 selective inhibitors which preferentially distribute to the brain and are relatively free from emesis in a range of animal models (Burgin et al. 2010).

Furthermore, there are examples of nonselective PDE4 inhibitors that have *in vivo* anti-inflammatory activity but are not emetogenic (Aoki et al. 2000, 2001; Gale et al. 2002). Similarly, the emetic profile of various PDE4 inhibitors (PMNPQ > R-rolipram > CT-2450) could not be explained by a preferred selectivity for PDE4D over PDE4A or PDE4B (Robichaud et al. 1999, 2002b). Some PDE4 inhibitors may preferentially partition within the area postrema in the CNS and thereby enhancing the inhibition of PDE4D in area postrema neurons and explaining the differences in the emetic potential of these drugs (Aoki et al. 2001; Robichaud et al. 2002a). Indeed, brain penetration by PMNPQ was 46-fold greater than CT-2450; however, the concentration of the “low emetic” PDE4 inhibitor, CT-2450, within the CNS was still 475-fold greater than the PDE4D inhibitory potency for CT-2450, and presumably still in sufficient concentrations to inhibit this enzyme. Moreover, the area postrema is not completely behind the blood–brain barrier and therefore accessible to free drug within the circulation (Gross et al. 1990). Whether differential partitioning of these inhibitors within the area postrema accounts for why some PDE4 inhibitors have a reduced emetic profile remains to be established. Alternatively, partial inhibition of PDE4D within the emetic center of the brain may not be sufficient to promote an emetogenic signal, although this degree of inhibition within inflammatory cells is sufficient enough to exert an *in vitro* anti-inflammatory effect (Burgin et al. 2010).

A number of preclinical studies have highlighted a number of other potential disadvantages to targeting PDE4, and these include the development of mesenteric vasculitis (Spina 2004), immunosuppression (Spina 2004), heart failure, and arrhythmia (Lehnart et al. 2005). However, none of these events appear to be realized in phase II and phase III clinical trials undertaken to date, at least with cilomilast and roflumilast. Similarly, slow release theophylline has been used for decades in the treatment of asthma and COPD and has not been associated with a number of these potentially adverse events despite being shown to cause mesenteric vasculitis preclinically in some models (Nyska et al. 1998; Ohta et al. 2004). It has also been suggested that PDE4 inhibitors may have pro-inflammatory properties, which is based on the finding that at very high doses, roflumilast (100 mg/kg) promoted the recruitment of neutrophils to the airways and this correlated with the release of IL-8 from cultured endothelial cells *in vitro* (McCluskie et al. 2006). This mechanism might explain why animals that have been chronically treated with high doses of PDE4 inhibitors document vasculitis. However, the concentrations required to achieve these untoward effects are at least 1,000 times greater than the ED₅₀ and EC₅₀ values reported for roflumilast against several *in vivo* biomarkers of inflammation and cell function *in vitro*, respectively (Bundschuh et al. 2001; Hatzelmann and Schudt 2001). It is unlikely that the plasma concentrations required to produce this purported pro-inflammatory effect could be achieved even with chronic dosing. Similarly, another study has shown that PDE4 inhibitors, at concentrations that are pharmacologically relevant, delay apoptosis of neutrophils and eosinophils, an effect that increased when combined with β_2 -agonists (Parkkonen et al. 2007). However, the extent to which these findings translate into the clinic is unclear, particularly as the beneficial effect of roflumilast in moderate-to-severe COPD

subjects was not compromised in patients taking long-acting β_2 -agonists (Fabbri et al. 2009). The clinical evidence suggests that PDE4 inhibitors suppress rather than exacerbate inflammation in the airways (Gamble et al. 2003; Grootendorst et al. 2007). Furthermore, there was no evidence of an increased incidence of pneumonia in patients with COPD clinically treated with roflumilast, which might be anticipated if PDE4 inhibitors interfere with host defense (Fabbri et al. 2009; Calverley et al. 2009). Interestingly, weight loss was an unexpected effect in subjects independent of any reports of gastrointestinal discomfort (Fabbri et al. 2009; Calverley et al. 2009) and could be a result of the well-recognized effect of elevating cAMP in adipocytes in promoting lipolysis since it has been previously reported that β_2 -agonists have a well-known lipolytic action. Mice deficient in PDE4D but not PDE4B have abnormal growth development as demonstrated by reduced fertility and litter size by female mice, reduced growth rates, and body weight in both sexes, the latter a consequence of a significant reduction in the weight of bone, muscle, and body organs (Jin et al. 1999; Jin and Conti 2002). Although the effect of these gene deficiencies were not studied on lipolysis per se, both PDE4B and PDE4D are present in rat adipocytes and inhibition of these enzymes promotes basal lipolysis (Wang and Edens 2007).

7 PDE4 Inhibition and the Future

Strategies at improving the risk/benefit ratio for PDE4 inhibition will be important if this drug class is to be widely used in the treatment of inflammatory diseases. The therapeutic window between the anti-inflammatory action of these drugs and side effects such as nausea and emesis is probably not wide enough for cilomilast, and may limit the use of roflumilast. It is of interest that there are a number of PDE4 inhibitors currently in development that appear to lack significant emetic action; these include oglemilast and IPL512602 (Boswell-Smith et al. 2006b), apremilast (Schafer et al. 2010), and the recently reported compounds produced by the Biotech company Decode (Burgin et al. 2010), but the molecular basis for this lack of emesis has not been published.

Most PDE4 inhibitors under development are designed for oral administration; however, the inhaled route would deliver a PDE4 inhibitor directly to target cells within the lung and thereby minimize systemic absorption and of course this is a widely accepted route of administration in pulmonary medicine as a way of minimizing systemic side effects with other drug classes. Both AWD 12-281 (*N*-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide) (Kuss et al. 2003) and UK-500,001 (Phillips et al. 2007; Vestbo et al. 2009) are examples of inhaled PDE4 inhibitors. Indeed, a 7-day treatment with the inhaled PDE4 inhibitor GSK256066 produced modest attenuation of both the early and the late asthmatic response to antigen challenge (Singh et al. 2010). It clearly should be possible to obtain anti-inflammatory activity by direct delivery of this drug class to the lung, although this does not always mean a complete loss of

emesis and nausea, as exemplified by the mixed PDE3/4 inhibitor zardaverine, which although inducing bronchodilation clinically also induced nausea (Brunnee et al. 1992).

Another approach might be the use of antisense oligodeoxynucleotides targeting PDE4, which could be delivered by the inhaled route. The positive results obtained in the successful targeting of the adenosine A1 receptor in a rabbit model of allergic inflammation (Nyce and Metzger 1997) illustrates the potential of this approach. A preclinical study has demonstrated that antisense oligonucleotides against mRNA for PDE4B/4D and PDE7 delivered topically to the lung by the endotracheal route of administration suppressed inflammation following 2 weeks of cigarette smoke exposure in mice (Fortin et al. 2009). The advantage of this technique is that side effects such as nausea and emesis are likely to be avoided. Alternatively, the recently identified allosteric modulators of PDE4 are proposed to confer lower emetic potential because despite only partially inhibiting PDE4 activity, the effect is nonetheless sufficient to inhibit inflammatory cell activity completely (Burgin et al. 2010). This is an important possibility that could be exploited in the discovery of nonemetic PDE4 inhibitors for the treatment of inflammatory diseases.

Another reason that targeting PDE4 alone may not fully resolve airway inflammation is the fact that there are other PDE types exist in structural and inflammatory cells in the lung (Table 1). Therefore, targeting multiple PDEs may be required for optimal anti-inflammatory action. For example, the macrophage is viewed as a critical cell type in the pathogenesis of COPD (Barnes 2008); however, the ability of these cells to release TNF α in response to endotoxin was only inhibited to a small degree by PDE4 inhibitors (Hatzelmann and Schudt 2001) and the potential functional involvement of PDE3 and PDE7 in these cells cannot be completely ignored. The inhibitory action of PDE4 inhibitors on the cellular activity of CD8⁺ T-lymphocytes and macrophages was significantly increased in the presence of PDE7 selective inhibitors (Smith et al. 2004). Similarly, combined PDE3 and PDE4 inhibitor in a single molecule offers the advantage of delivering a bronchodilator and anti-inflammatory substance. Moreover, it is likely that retention of the inhibitor within the lung may be required in order to maintain anti-inflammatory activity with the airways (Boswell-Smith et al. 2006a).

8 Conclusion

A number of clinical trials assessing the efficacy of PDE4 inhibitors for the treatment of inflammatory disease including asthma, COPD, atopic dermatitis, and psoriasis have demonstrated moderate success. However, the dose-limiting side effects such as nausea, emesis, and headache potentially limit the utility of these drugs. Importantly, there are examples of PDE4 inhibitors that have low emetogenic potential, although the molecular basis of this phenomenon remains to be established. Other strategies include topical delivery (e.g., inhalation for administration to the lung; direct application to the skin), development of subtype

selective PDE4 inhibitors, use of mixed PDE inhibitors, interference with PDE4 activation, targeting proteins that are involved in locating PDE4 to specific micro-cellular domains and finally the potential for use of antisense oligonucleotides may offer another solution to the problem of targeting PDE4 in inflammation is a cause for optimism.

Conflict of Interest. The authors are consultants for Veronapharma plc who are developing RPL554 as a novel inhaled PDE3/4 inhibitor for the treatment of inflammatory airways disease. CP is also a founder and has equity in Veronapharma plc.

References

- Abraham C, Cho JH (2009) Inflammatory bowel disease. *N Engl J Med* 361:2066–2078
- Akama T, Baker SJ, Zhang YK, Hernandez V, Zhou H, Sanders V, Freund Y, Kimura R, Maples KR, Plattner JJ (2009) Discovery and structure-activity study of a novel benzoxaborole anti-inflammatory agent (AN2728) for the potential topical treatment of psoriasis and atopic dermatitis. *Bioorg Med Chem Lett* 19:2129–2132
- Aoki M, Kobayashi M, Ishikawa J, Saita Y, Terai Y, Takayama K, Miyata K, Yamada T (2000) A novel phosphodiesterase type 4 inhibitor, YM976 (4-(3-chlorophenyl)-1, 7-diethylpyrido [2, 3-d]pyrimidin-2(1H)-one), with little emetogenic activity. *J Pharmacol Exp Ther* 295: 255–260
- Aoki M, Fukunaga M, Sugimoto T, Hirano Y, Kobayashi M, Honda K, Yamada T (2001) Studies on mechanisms of low emetogenicity of YM976, a novel phosphodiesterase type 4 inhibitor. *J Pharmacol Exp Ther* 298:1142–1149
- Ariga M, Neitzert B, Nakae S, Mottin G, Bertrand C, Pruniaux MP, Jin SL, Conti M (2004) Nonredundant function of phosphodiesterases 4D and 4B in neutrophil recruitment to the site of inflammation. *J Immunol* 173:7531–7538
- Banner KH, Press NJ (2009) Dual PDE3/4 inhibitors as therapeutic agents for chronic obstructive pulmonary disease. *Br J Pharmacol* 157:892–906
- Barber R, Baillie GS, Bergmann R, Shepherd MC, Sepper R, Houslay MD, Heeke GV (2004) Differential expression of PDE4 cAMP phosphodiesterase isoforms in inflammatory cells of smokers with COPD, smokers without COPD, and nonsmokers. *Am J Physiol Lung Cell Mol Physiol* 287:L332–L343
- Barnes PJ (2008) The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 118:3546–3556
- Barnes PJ, Adcock IM, Ito K (2005) Histone acetylation and deacetylation: importance in inflammatory lung diseases. *Eur Respir J* 25:552–563
- Baumer W, Hoppmann J, Rundfeldt C, Kietzmann M (2007) Highly selective phosphodiesterase 4 inhibitors for the treatment of allergic skin diseases and psoriasis. *Inflamm Allergy Drug Targets* 6:17–26
- Boolell M, Gopi-Attee S, Gingell JC, Allen MJ (1996) Sildenafil, a novel effective oral therapy for male erectile dysfunction. *Br J Urol* 78:257–261
- Boswell-Smith V, Spina D, Oxford AW, Comer MB, Seeds EA, Page CP (2006a) The pharmacology of two novel long-acting phosphodiesterase 3/4 inhibitors, RPL554 [9, 10-dimethoxy-2(2, 4, 6-trimethylphenylimino)-3-(n-carbamoyl-2-aminoethyl)-3, 4, 6, 7-tetrahydro-2H-pyrimido[6, 1-a] isoquinolin-4-one] and RPL565 [6, 7-dihydro-2-(2, 6-diisopropylphenoxy)-9, 10-dimethoxy-4H-pyrimido[6, 1-a] soquinolin-4-one]. *J Pharmacol Exp Ther* 318:840–848
- Boswell-Smith V, Spina D, Page CP (2006b) Phosphodiesterase inhibitors. *Br J Pharmacol* 147 (Suppl 1):S252–S257

- Bousquet J, Aubier M, Sastre J, Izquierdo JL, Adler LM, Hofbauer P, Rost KD, Harnest U, Kroemer B, Albrecht A, Bredenbroeker D (2006) Comparison of roflumilast, an oral anti-inflammatory, with beclomethasone dipropionate in the treatment of persistent asthma. *Allergy* 61:72–78
- Brunnee T, Engelstatter R, Steinijs VW, Kunkel G (1992) Bronchodilatory effect of inhaled zardaverine, a phosphodiesterase III and IV inhibitor, in patients with asthma. *Eur Respir J* 5:982–985
- Bundschuh DS, Eltze M, Barsig J, Wollin L, Hatzelmann A, Beume R (2001) In vivo efficacy in airway disease models of roflumilast, a novel orally active PDE4 inhibitor. *J Pharmacol Exp Ther* 297:280–290
- Bureau Y, Handa M, Zhu Y, Laliberte F, Moore CS, Liu S, Huang Z, Macdonald D, Xu DG, Robertson GS (2006) Neuroanatomical and pharmacological assessment of Fos expression induced in the rat brain by the phosphodiesterase-4 inhibitor 6-(4-pyridylmethyl)-8-(3-nitrophenyl) quinoline. *Neuropharmacology* 51:974–985
- Burgin AB, Magnusson OT, Singh J, Witte P, Staker BL, Bjornsson JM, Thorsteinsdottir M, Hrafnisdottir S, Hagen T, Kiselyov AS, Stewart LJ, Gurney ME (2010) Design of phosphodiesterase 4D (PDE4D) allosteric modulators for enhancing cognition with improved safety. *Nat Biotechnol* 28:63–70
- Butcher RW, Sutherland EW (1962) Adenosine 3', 5'-phosphate in biological materials. I. Purification and properties of cyclic 3', 5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3', 5'-phosphate in human urine. *J Biol Chem* 237:1244–1250
- Calverley PM, Sanchez-Toril F, McIvor A, Teichmann P, Bredenbroeker D, Fabbri LM (2007) Effect of 1-year treatment with roflumilast in severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 176:154–161
- Calverley PM, Rabe KF, Goehring UM, Kristiansen S, Fabbri LM, Martinez FJ (2009) Roflumilast in symptomatic chronic obstructive pulmonary disease: two randomised clinical trials. *Lancet* 374:685–694
- Card GL, England BP, Suzuki Y, Fong D, Powell B, Lee B, Luu C, Tabrizizad M, Gillette S, Ibrahim PN, Artis DR, Bollag G, Milburn MV, Kim SH, Schlessinger J, Zhang KY (2004) Structural basis for the activity of drugs that inhibit phosphodiesterases. *Structure* 12:2233–2247
- Carpenter DO, Briggs DB, Knox AP, Strominger N (1988) Excitation of area postrema neurones by transmitters, peptides and cyclic nucleotides. *J Neurophysiol* 59:358–369
- Chan SC, Reifsnnyder D, Beavo JA, Hanifin JM (1993) Immunochemical characterization of the distinct monocyte cyclic AMP-phosphodiesterase from patients with atopic dermatitis. *J Allergy Clin Immunol* 91:1179–1188
- Cherry JA, Davis RL (1999) Cyclic AMP phosphodiesterases are localized in regions of the mouse brain associated with reinforcement, movement and affect. *J Comp Neurol* 407:287–301
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- David M, Zech K, Seiberling M, Weimar C, Roflumilast LTD (2004) a novel, oral, selective PDE4 inhibitor, shows high absolute bioavailability. *J Allergy Clin Immunol* 113:S220–S221, Ref Type: Abstract
- Dunkern TR, Feurstein D, Rossi GA, Sabatini F, Hatzelmann A (2007) Inhibition of TGF-beta induced lung fibroblast to myofibroblast conversion by phosphodiesterase inhibiting drugs and activators of soluble guanylyl cyclase. *Eur J Pharmacol* 572:12–22
- Essayan DM, Kagey-Sobotka A, Lichtenstein LM, Huang S-K (1997) Differential regulation of human antigen-specific Th1 and Th2 lymphocyte responses by isozyme selective cyclic nucleotide phosphodiesterase inhibitors. *J Pharmacol Exp Ther* 282:505–512
- Fabbri LM, Calverley PM, Izquierdo-Alonso JL, Bundschuh DS, Brose M, Martinez FJ, Rabe KF (2009) Roflumilast in moderate-to-severe chronic obstructive pulmonary disease treated with longacting bronchodilators: two randomised clinical trials. *Lancet* 374:695–703
- Fortin M, D'Anjou H, Higgins ME, Gougeon J, Aube P, Moktefi K, Mouissi S, Seguin S, Seguin R, Renzi PM, Paquet L, Ferrari N (2009) A multi-target antisense approach against PDE4 and PDE7 reduces smoke-induced lung inflammation in mice. *Respir Res* 10:39

- Fuhrmann M, Jahn H-U, Seybold J, Neurohr C, Barnes PJ, Hippenstiel S, Kraemer HJ, Suttorp N (1999) Identification and function of cyclic nucleotide phosphodiesterase isoenzymes in airway epithelial cells. *Am J Respir Cell Mol Biol* 20:292–302
- Gale DD, Landells LJ, Spina D, Miller AJ, Smith K, Nichols T, Rotshteyn Y, Tonelli A, Lacouture P, Burch RM, Page CP, O'Connor BJ (2002) Pharmacokinetic and pharmacodynamic profile following oral administration of the phosphodiesterase (PDE)4 inhibitor V11294A in healthy volunteers. *Br J Clin Pharmacol* 54:478–484
- Gamble E, Grootendorst DC, Brightling CE, Troy S, Qiu Y, Zhu J, Parker D, Matin D, Majumdar S, Vignola AM, Kroegel C, Morell F, Hansel TT, Rennard SI, Compton C, Amit O, Tat T, Edelson J, Pavord ID, Rabe KF, Barnes NC, Jeffery PK (2003) Antiinflammatory effects of the phosphodiesterase-4 inhibitor cilomilast (Ariflo) in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 168:976–982
- Gantner F, Kupferschmidt R, Schudt C, Wendel A, Hatzelmann A (1997a) In vitro differentiation of human monocytes to macrophages: Change of PDE profile and its relationship to suppression of tumour necrosis factor-alpha release by PDE inhibitors. *Br J Pharmacol* 121:221–231
- Gantner F, Tenor H, Gekeler V, Schudt C, Wendel A, Hatzelmann A (1997b) Phosphodiesterase profiles of highly purified human peripheral blood leukocyte populations from normal and atopic individuals: a comparative study. *J Allergy Clin Immunol* 100:527–535
- Gantner F, Gotz C, Gekeler V, Schudt C, Wendel A, Hatzelmann A (1998) Phosphodiesterase profile of human B lymphocytes from normal and atopic donors and the effects of PDE inhibition on B cell proliferation. *Br J Pharmacol* 123:1031–1038
- Gottlieb AB, Strober B, Krueger JG, Rohane P, Zeldis JB, Hu CC, Kipnis C (2008) An open-label, single-arm pilot study in patients with severe plaque-type psoriasis treated with an oral anti-inflammatory agent, apremilast. *Curr Med Res Opin* 24:1529–1538
- Grootendorst DC, Gauw SA, Verhoosel RM, Sterk PJ, Hoppers JJ, Bredenbroeker D, Bethke TD, Hiemstra PS, Rabe KF (2007) Reduction in sputum neutrophil and eosinophil numbers by the PDE4 inhibitor roflumilast in patients with COPD. *Thorax* 62:1081–1087
- Gross PM, Wall KM, Pang JJ, Shaver SW, Wainman DS (1990) Microvascular specializations promoting rapid interstitial solute dispersion in nucleus tractus solitarius. *Am J Physiol* 259: R1131–R1138
- Haddad JJ, Land SC, Tarnow-Mordi WO, Zembala M, Kowalczyk D, Lauterbach R (2002) Immunopharmacological potential of selective phosphodiesterase inhibition. I. Differential regulation of lipopolysaccharide-mediated proinflammatory cytokine (interleukin-6 and tumor necrosis factor-alpha) biosynthesis in alveolar epithelial cells. *J Pharmacol Exp Ther* 300: 559–566
- Hansen G, Jin S, Umetsu DT, Conti M (2000) Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. *Proc Natl Acad Sci USA* 97:6751–6756
- Harbinson PL, MacLeod D, Hawksworth R, O'Toole S, Sullivan PJ, Heath P, Kilfeather S, Page CP, Costello J, Holgate ST, Lee TH (1997) The effect of a novel orally active selective PDE4 isoenzyme inhibitor (CDP840) on allergen-induced responses in asthmatic subjects. *Eur Respir J* 10:1008–1014
- Hatzelmann A, Schudt C (2001) Anti-inflammatory and immunomodulatory potential of the novel PDE4 inhibitor roflumilast in vitro. *J Pharmacol Exp Ther* 297:267–279
- Heystek HC, Thierry AC, Soulard P, Moulon C (2003) Phosphodiesterase 4 inhibitors reduce human dendritic cell inflammatory cytokine production and Th1-polarizing capacity. *Int Immunol* 15:827–835
- Himes BE, Hunninghake GM, Baurley JW, Rafaels NM, Sleiman P, Strachan DP, Wilk JB, Willis-Owen SA, Klanderman B, Lasky-Su J, Lazarus R, Murphy AJ, Soto-Quiros ME, Avila L, Beaty T, Mathias RA, Ruczinski I, Barnes KC, Celedon JC, Cookson WO, Gauderman WJ, Gilliland FD, Hakonarson H, Lange C, Moffatt MF, O'Connor GT, Raby BA, Silverman EK, Weiss ST (2009) Genome-wide association analysis identifies PDE4D as an asthma-susceptibility gene. *Am J Hum Genet* 84:581–593

- Homma S, Sakamoto T, Hegab AE, Saitoh W, Nomura A, Ishii Y, Morishima Y, Iizuka T, Kiwamoto T, Matsuno Y, Massoud HH, Massoud HM, Hassanein KM, Sekizawa K (2006) Association of phosphodiesterase 4D gene polymorphisms with chronic obstructive pulmonary disease: relationship to interleukin 13 gene polymorphism. *Int J Mol Med* 18:933–939
- Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 35:91–100
- Houslay MD, Schafer P, Zhang KY (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10:1503–1519
- Houslay MD, Baillie GS, Maurice DH (2007) cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. *Circ Res* 100:950–966
- Huston E, Lynch MJ, Mohamed A, Collins DM, Hill EV, MacLeod R, Krause E, Baillie GS, Houslay MD (2008) EPAC and PKA allow cAMP dual control over DNA-PK nuclear translocation. *Proc Natl Acad Sci USA* 105:12791–12796
- Ichikawa H, Okamoto S, Kamada N, Nagamoto H, Kitazume MT, Kobayashi T, Chinen H, Hisamatsu T, Hibi T (2008) Tetomilast suppressed production of proinflammatory cytokines from human monocytes and ameliorated chronic colitis in IL-10-deficient mice. *Inflamm Bowel Dis* 14:1483–1490
- Jin SL, Conti M (2002) Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF- α responses. *Proc Natl Acad Sci USA* 99:7628–7633
- Jin SL, Richard FJ, Kuo WP, D'Ercole AJ, Conti M (1999) Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice. *Proc Natl Acad Sci USA* 96:11998–12003
- Jones NA, Boswell-Smith V, Lever R, Page CP (2005) The effect of selective phosphodiesterase isoenzyme inhibition on neutrophil function in vitro. *Pulm Pharmacol Ther* 18:93–101
- Jones NA, Leport M, Holand T, Vos T, Morgan M, Fink M, Pruniaux MP, Berthelier C, O'Connor BJ, Bertrand C, Page CP (2007) Phosphodiesterase (PDE) 7 in inflammatory cells from patients with asthma and COPD. *Pulm Pharmacol Ther* 20:60–68
- Kanehiro A, Ikemura T, Makela MJ, Lahn M, Joetham A, Dakhama A, Gelfand EW (2001) Inhibition of phosphodiesterase 4 attenuates airway hyperresponsiveness and airway inflammation in a model of secondary allergen challenge. *Am J Respir Crit Care Med* 163:173–184
- Keshavarzian A, Mutlu E, Guzman JP, Forsyth C, Banan A (2007) Phosphodiesterase 4 inhibitors and inflammatory bowel disease: emerging therapies in inflammatory bowel disease. *Expert Opin Investig Drugs* 16:1489–1506
- Kobayashi K, Suda T, Manabe H, Miki I (2007) Administration of PDE4 inhibitors suppressed the pannus-like inflammation by inhibition of cytokine production by macrophages and synovial fibroblast proliferation. *Mediat Inflamm* 2007:58901
- Kohyama T, Liu X, Zhu YK, Wen FQ, Wang HJ, Fang Q, Kobayashi T, Rennard SI (2002) Phosphodiesterase 4 inhibitor cilomilast inhibits fibroblast-mediated collagen gel degradation induced by tumor necrosis factor- α and neutrophil elastase. *Am J Respir Cell Mol Biol* 27:487–494
- Kranz M, Wall M, Evans B, Miah A, Ballantine S, Delves C, Dombroski B, Gross J, Schneck J, Villa JP, Neu M, Somers DO (2009) Identification of PDE4B Over 4D subtype-selective inhibitors revealing an unprecedented binding mode. *Bioorg Med Chem* 17:5336–5341
- Kumar RK, Herbert C, Thomas PS, Wollin L, Beume R, Yang M, Webb DC, Foster PS (2003) Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. *J Pharmacol Exp Ther* 307:349–355
- Kung TT, Crawley Y, Luo B, Young S, Kreutner W, Chapman RW (2000) Inhibition of pulmonary eosinophilia and airway hyperresponsiveness in allergic mice by rolipram: involvement of endogenously released corticosterone and catecholamines. *Br J Pharmacol* 130:457–463
- Kuss H, Hoefgen N, Johanssen S, Kronbach T, Rundfeldt C (2003) In vivo efficacy in airway disease models of N-(3, 5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyo

- xylic acid amide (AWD 12–281), a selective phosphodiesterase 4 inhibitor for inhaled administration. *J Pharmacol Exp Ther* 307:373–385
- Lamontagne S, Meadows E, Luk P, Normandin D, Muise E, Boulet L, Pon DJ, Robichaud A, Robertson GS, Metters KM, Nantel F (2001) Localization of phosphodiesterase-4 isoforms in the medulla and nodose ganglion of the squirrel monkey. *Brain Res* 920:84–96
- Landells LJ, Szilagy CM, Jones NA, Banner KH, Allen JM, Doherty A, O'Connor BJ, Spina D, Page CP (2001) Identification and quantification of phosphodiesterase 4 subtypes in CD4 and CD8 lymphocytes from healthy and asthmatic subjects. *Br J Pharmacol* 133:722–729
- Leclerc O, Lagente V, Planquois JM, Berthelie C, Artola M, Eichholtz T, Bertrand CP, Schmidlin F (2006) Involvement of MMP-12 and phosphodiesterase type 4 in cigarette smoke-induced inflammation in mice. *Eur Respir J* 27:1102–1109
- Lehnart SE, Wehrens XH, Reiken S, Warriar S, Belevych AE, Harvey RD, Richter W, Jin SL, Conti M, Marks AR (2005) Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. *Cell* 123:25–35
- Louw C, Williams Z, Venter L, Leichtl S, Schmid-Wirlitsch C, Bredenbrocker D, Bardin PG (2007) Roflumilast, a phosphodiesterase 4 inhibitor, reduces airway hyperresponsiveness after allergen challenge. *Respiration* 74:411–417
- Lu S, Liu N, Dass SB, Reiss TF, Knorr BA (2009) Randomized, placebo-controlled study of a selective PDE4 inhibitor in the treatment of asthma. *Respir Med* 103:342–347
- Ma R, Yang BY, Wu CY (2008) A selective phosphodiesterase 4 (PDE4) inhibitor Z1-n-91 suppresses IL-17 production by human memory Th17 cells. *Int Immunopharmacol* 8:1408–1417
- Manning CD, Burman M, Christensen SB, Cieslinski LB, Essayan DM, Grous M, Torphy TJ, Barnette MS (1999) Suppression of human inflammatory cell function by subtype-selective PDE4 inhibitors correlates with inhibition of PDE4A and PDE4B. *Br J Pharmacol* 128:1393–1398
- Martin-Chouly CA, Astier A, Jacob C, Pruniaux MP, Bertrand C, Lagente V (2004) Modulation of matrix metalloproteinase production from human lung fibroblasts by type 4 phosphodiesterase inhibitors. *Life Sci* 75:823–840
- Martorana PA, Beume R, Lucattelli M, Wollin L, Lungarella G (2005) Roflumilast fully prevents emphysema in mice chronically exposed to cigarette smoke. *Am J Respir Crit Care Med* 172:848–853
- McCahill AC, Huston E, Li X, Houslay MD (2008) PDE4 associates with different scaffolding proteins: modulating interactions as treatment for certain diseases. *Handb Exp Pharmacol* (186): 125–166
- McCluskie K, Klein U, Linnevers C, Ji YH, Yang A, Husfeld C, Thomas GR (2006) Phosphodiesterase type 4 inhibitors cause proinflammatory effects in vivo. *J Pharmacol Exp Ther* 319:468–476
- Mehats C, Jin SL, Wahlstrom J, Law E, Umetsu DT, Conti M (2003) PDE4D plays a critical role in the control of airway smooth muscle contraction. *FASEB J* 17:1831–1841
- Millar JK, Mackie S, Clapcote SJ, Murdoch H, Pickard BS, Christie S, Muir WJ, Blackwood DH, Roder JC, Houslay MD, Porteous DJ (2007) Disrupted in schizophrenia 1 and phosphodiesterase 4B: towards an understanding of psychiatric illness. *J Physiol* 584:401–405
- Nazarian R, Weinberg JM (2009) AN-2728, a PDE4 inhibitor for the potential topical treatment of psoriasis and atopic dermatitis. *Curr Opin Investig Drugs* 10:1236–1242
- Nestle FO, Kaplan DH, Barker J (2009) Psoriasis. *N Engl J Med* 361:496–509
- Noh AL, Yang M, Lee JM, Park H, Lee DS, Yim M (2009) Phosphodiesterase 3 and 4 negatively regulate receptor activator of nuclear factor-kappaB ligand-mediated osteoclast formation by prostaglandin E2. *Biol Pharm Bull* 32:1844–1848
- Nyce JW, Metzger WJ (1997) DNA antisense therapy for asthma in an animal model. *Nature* 385:721–725
- Nyska A, Herbert RA, Chan PC, Haseman JK, Hailey JR (1998) Theophylline-induced mesenteric periarteritis in F344/N rats. *Arch Toxicol* 72:731–737

- O'Shaughnessy MJ, Chen ZM, Gramaglia I, Taylor PA, Panoskaltsis-Mortari A, Vogtenhuber C, Palmer E, Grader-Beck T, Boussiotis VA, Blazar BR (2007) Elevation of intracellular cyclic AMP in alloreactive CD4(+) T Cells induces alloantigen-specific tolerance that can prevent GVHD lethality in vivo. *Biol Blood Marrow Transplant* 13:530–542
- Ohta K, Fukuchi Y, Grouse L, Mizutani R, Rabe KF, Rennard SI, Zhong NS (2004) A prospective clinical study of theophylline safety in 3810 elderly with asthma or COPD. *Respir Med* 98:1016–1024
- Papakostantinou E, Xenos K, Markantonis SL, Druska S, Stratigos A, Katsambas A (2005) Efficacy of 2 weeks' application of theophylline ointment in psoriasis vulgaris. *J Dermatol Treat* 16:169–170
- Parkkonen J, Hasala H, Moilanen E, Giembycz MA, Kankaanranta H (2007) Phosphodiesterase 4 inhibitors delay human eosinophil and neutrophil apoptosis in the absence and presence of salbutamol. *Pulm Pharmacol Ther* 21:499–506
- Perez-Torres S, Miro X, Palacios JM, Cortes R, Puigdomenech P, Mengod G (2000) Phosphodiesterase type 4 isozymes expression in human brain examined by in situ hybridization histochemistry and [³H]rolipram binding autoradiography. Comparison with monkey and rat brain. *J Chem Neuroanat* 20:349–374
- Peter D, Jin SL, Conti M, Hatzelmann A, Zitt C (2007) Differential expression and function of phosphodiesterase 4 (PDE4) subtypes in human primary CD4+ T cells: predominant role of PDE4D. *J Immunol* 178:4820–4831
- Phillips P, Bennetts M, Banner K, Ward J, Wessels D, Fuhr R (2007) The PDE4 inhibitor UK-500,001 does not significantly inhibit airway responses to allergen and histamine. *Eur Resp J*: 490s. Ref Type: Abstract
- Rabe KF, Bateman ED, O'Donnell D, Witte S, Bredenbrocker D, Bethke TD (2005) Roflumilast – an oral anti-inflammatory treatment for chronic obstructive pulmonary disease: a randomised controlled trial. *Lancet* 366:563–571
- Rennard SI (2004) Treatment of stable chronic obstructive pulmonary disease. *Lancet* 364:791–802
- Rennard SI, Schachter N, Streck M, Rickard K, Amit O (2006) Cilomilast for COPD: results of a 6-month, placebo-controlled study of a potent, selective inhibitor of phosphodiesterase 4. *Chest* 129:56–66
- Robichaud A, Tattersall FD, Choudhury I, Rodger IW (1999) Emesis induced by inhibitors of type IV cyclic nucleotide phosphodiesterase (PDE IV) in the ferret. *Neuropharmacology* 38:289–297
- Robichaud A, Savoie C, Stamatiou PB, Tattersall FD, Chan CC (2001) PDE4 inhibitors induce emesis in ferrets via a noradrenergic pathway. *Neuropharmacology* 40:262–269
- Robichaud A, Savoie C, Stamatiou PB, Lachance N, Jolicoeur P, Rasori R, Chan CC (2002a) Assessing the emetic potential of PDE4 inhibitors in rats. *Br J Pharmacol* 135:113–118
- Robichaud A, Stamatiou PB, Jin SL, Lachance N, Macdonald D, Laliberte F, Liu S, Huang Z, Conti M, Chan CC (2002b) Deletion of phosphodiesterase 4D in mice shortens alpha(2)-adrenoceptor-mediated anesthesia, a behavioral correlate of emesis. *J Clin Invest* 110: 1045–1052
- Roth BL, Sheffler DJ, Kroeze WK (2004) Magic shotguns versus magic bullets: selectively non-selective drugs for mood disorders and schizophrenia. *Nat Rev Drug Discov* 3:353–359
- Salvi SS, Barnes PJ (2009) Chronic obstructive pulmonary disease in non-smokers. *Lancet* 374:733–743
- Sanz MJ, Cortijo J, Taha MA, Cerda-Nicolas M, Schatton E, Burgbacher B, Klar J, Tenor H, Schudt C, Issekutz AC, Hatzelmann A, Morcillo EJ (2007) Roflumilast inhibits leukocyte-endothelial cell interactions, expression of adhesion molecules and microvascular permeability. *Br J Pharmacol* 152:481–492
- Schafer P, Parton A, Gandhi A, Capone L, Adams M, Wu L, Bartlett J, Loveland M, Gilhar A, Cheung YF, Baillie G, Houslay M, Man HW, Muller G, Stirling D (2010) Apremilast, a cAMP phosphodiesterase-4 inhibitor, demonstrates anti-inflammatory activity in vitro and in a model of psoriasis. *Br J Pharmacol* 159:842–855

- Schreiber S, Keshavarzian A, Isaacs KL, Schollenberger J, Guzman JP, Orlandi C, Hanauer SB (2007) A randomized, placebo-controlled, phase II study of tetomilast in active ulcerative colitis. *Gastroenterology* 132:76–86
- Shichijo M, Inagaki N, Nakai N, Kimata M, Nakahata T, Serizawa I, Iikura Y, Saito H, Nagai H (1998) The effects of anti-asthma drugs on mediator release from cultured human mast cells. *Clin Exp Allergy* 28:1228–1236
- Shore A, Dosch H, Gelfand EW (1978) Induction and separation of antigen-dependent T helper and T suppressor cells in man. *Nature* 274:586–587
- Singh D, Petavy F, Macdonald AJ, Lazaar AL, O'Connor BJ (2010) The inhaled phosphodiesterase 4 inhibitor GSK256066 reduces allergen challenge responses in asthma. *Respir Res* 11:26–35
- Smith SJ, Brookes-Fazakerley S, Donnelly LE, Barnes PJ, Barnette MS, Giembycz MA (2003) Ubiquitous expression of phosphodiesterase 7A in human proinflammatory and immune cells. *Am J Physiol Lung Cell Mol Physiol* 284:L279–L289
- Smith SJ, Cieslinski LB, Newton R, Donnelly LE, Fenwick PS, Nicholson AG, Barnes PJ, Barnette MS, Giembycz MA (2004) Discovery of BRL 50481 [3-(N, N-dimethylsulfonamido)-4-methyl-nitrobenzene], a selective inhibitor of phosphodiesterase 7: in vitro studies in human monocytes, lung macrophages, and CD8+ T-lymphocytes. *Mol Pharmacol* 66:1679–1689
- Spina D (2003) Phosphodiesterase-4 inhibitors in the treatment of inflammatory lung disease. *Drugs* 63:2575–2594
- Spina D (2004) The potential of PDE4 inhibitors in respiratory disease. *Curr Drug Targets Inflamm Allergy* 3:231–236
- Spina D (2008) PDE4 inhibitors: current status. *Br J Pharmacol* 155:308–315
- Spina D, Harrison S, Page CP (1995) Regulation by phosphodiesterase isoenzymes of non-adrenergic non-cholinergic contraction in guinea-pig isolated main bronchus. *Br J Pharmacol* 116:2334–2340
- Sullivan P, Bekir S, Jaffar Z, Page C, Jeffery P, Costello J (1994) Anti-inflammatory effects of low-dose oral theophylline in atopic asthma [published erratum appears in *Lancet* 1994 Jun 11; 343(8911):1512]. *Lancet* 343:1006–1008
- Takahashi M, Terwilliger R, Lane C, Mezes PS, Conti M, Duman RS (1999) Chronic antidepressant administration increases the expression of cAMP-specific phosphodiesterase 4A and 4B isoforms. *J Neurosci* 19:610–618
- Taylor PC, Feldmann M (2009) Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis. *Nat Rev Rheumatol* 5:578–582
- Tenor H, Hedbom E, Hauselmann HJ, Schudt C, Hatzelmann A (2002) Phosphodiesterase isoenzyme families in human osteoarthritis chondrocytes—functional importance of phosphodiesterase 4. *Br J Pharmacol* 135:609–618
- Timmer W, Leclerc V, Birraux G, Neuhauser M, Hatzelmann A, Bethke T, Wurst W (2002) The new phosphodiesterase 4 inhibitor roflumilast is efficacious in exercise-induced asthma and leads to suppression of LPS-stimulated TNF-alpha ex vivo. *J Clin Pharmacol* 42:297–303
- van Schalkwyk E, Strydom K, Williams Z, Venter L, Leichtl S, Schmid-Wirlitsch C, Bredenkroter D, Bardin PG (2005) Roflumilast, an oral, once-daily phosphodiesterase 4 inhibitor, attenuates allergen-induced asthmatic reactions. *J Allergy Clin Immunol* 116:292–298
- Vestbo J, Tan L, Atkinson G, Ward J (2009) A controlled trial of 6-weeks' treatment with a novel inhaled phosphodiesterase type-4 inhibitor in COPD. *Eur Respir J* 33:1039–1044
- Wang H, Edens NK (2007) mRNA expression and antilipolytic role of phosphodiesterase 4 in rat adipocytes in vitro. *J Lipid Res* 48:1099–1107
- Wang H, Peng MS, Chen Y, Geng J, Robinson H, Houslay MD, Cai J, Ke H (2007) Structures of the four subfamilies of phosphodiesterase-4 provide insight into the selectivity of their inhibitors. *Biochem J* 408:193–201
- Weston MC, Anderson N, Peachell PT (1997) Effects of phosphodiesterase inhibitors on human lung mast cell and basophil function. *Br J Pharmacol* 121:287–295

- Wright RK, Mandy SH, Halprin KM, Hsia SL (1973) Defects and deficiency of adenylyl cyclase in psoriatic skin. *Arch Dermatol* 107:47–53
- Xu RX, Hassell AM, Vanderwall D, Lambert MH, Holmes WD, Luther MA, Rocque WJ, Milburn MV, Zhao Y, Ke H, Nolte RT (2000) Atomic structure of PDE4: insights into phosphodiesterase mechanism and specificity. *Science* 288:1822–1825
- Yao W, Tian XY, Chen J, Setterberg RB, Lundy MW, Chmielzowski P, Froman CA, Jee WS (2007) Rolipram, a phosphodiesterase 4 inhibitor, prevented cancellous and cortical bone loss by inhibiting endosteal bone resorption and maintaining the elevated periosteal bone formation in adult ovariectomized rats. *J Musculoskelet Neuronal Interact* 7:119–130
- Zocchi MR, Pardi R, Gromo G, Ferrero E, Ferrero ME, Besana C, Rugarli C (1985) Theophylline induced non specific suppressor activity in human peripheral blood lymphocytes. *J Immunopharmacol* 7:217–234

Harnessing the Clinical Efficacy of Phosphodiesterase 4 Inhibitors in Inflammatory Lung Diseases: Dual-Selective Phosphodiesterase Inhibitors and Novel Combination Therapies

Mark A. Giembycz and Robert Newton

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Abstract Phosphodiesterase (PDE) 4 inhibitors have been in development as a novel anti-inflammatory therapy for more than 20 years, with asthma and chronic obstructive pulmonary disease (COPD) being primary indications. Despite initial optimism, only one selective PDE4 inhibitor, roflumilast (*Daxas*[®]), has been approved for use in humans and available in Canada and the European Union in 2011 for the treatment of a specific population of patients with severe COPD. In many other cases, the development of PDE4 inhibitors of various structural classes has been discontinued due to lack of efficacy and/or dose-limiting adverse events.

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Indeed, for many of these compounds, it is likely that the maximum tolerated dose is either subtherapeutic or at the very bottom of the efficacy dose–response curve. Thus, a significant ongoing challenge that faces the pharmaceutical industry is to synthesize compounds with therapeutic ratios that are superior to roflumilast. Several strategies are being considered, but clinically effective compounds with an optimal pharmacophore have not, thus far, been reported. In this chapter, alternative means of harnessing the clinical efficacy of PDE4 inhibitors are described. These concepts are based on the assumption that additive or synergistic anti-inflammatory effects can be produced with inhibitors that target either two or more PDE families or with a PDE4 inhibitor in combination with other anti-inflammatory drugs such as a glucocorticoid.

Keywords Airway inflammation · Asthma · cAMP · Chronic obstructive pulmonary disease · Combination therapies · Gene transactivation · Glucocorticoids · Long-acting β_2 -adrenoceptor agonists · Nuclear hormone receptors · Phosphodiesterase 4

Abbreviations

AHR	Airway hyperresponsiveness
AP	Activator protein
AR	Androgen receptor
BP	Blood pressure
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
FEV ₁	Forced expiratory volume in 1 s
GILZ	Glucocorticoid-induced leucine zipper
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HDAC	Histone deacetylase
HPV	Hypoxic pulmonary vasoconstriction
HR	Heart rate
ICS	Inhaled corticosteroid
IL	Interleukin
LABA	Long-acting β_2 -adrenoceptor agonist
LVP	Left ventricular pressure
MKP	Mitogen-activated protein kinase phosphatase
MR	Mineralocorticoid receptor
NF κ B	Nuclear factor-kappaB
PDE	Phosphodiesterase
PH	Pulmonary hypertension
PHA	Phytohemagglutinin
PKA	cAMP-dependent protein kinase
Ppa	Pulmonary artery pressure

PR	Progesterone receptor
PVR	Pulmonary vascular resistance
RAR/RXR	Retinoic acid receptors
SABA	Short-acting β_2 -adrenoceptor agonist

1 Introduction

Since the early 1990s, there has been considerable interest in phosphodiesterase (PDE) 4 as an intracellular target that could be exploited to therapeutic advantage for a multitude of chronic inflammatory diseases, with asthma and chronic obstructive pulmonary disease (COPD) being primary indications (Burnouf and Pruniaux 2002; Doherty 1999; Dyke and Montana 2002; Giembycz 2001; Houslay et al. 2005; Huang et al. 2001; Souness et al. 2000; Torphy 1998). Although targeting PDE4 with small molecule inhibitors is based on a conceptually robust hypothesis (see references in Giembycz 2000), dose-limiting adverse events that include nausea and vomiting have impeded their clinical development (Burnouf et al. 1998; Giembycz 2006). These undesirable effects are due to the inhibition of PDE4 in nontarget tissues (Duplantier et al. 1996), and a fundamental challenge that is still to be adequately met by the pharmaceutical industry is to synthesize compounds with improved therapeutic ratios. Several strategies (not discussed here) are being considered to dissociate the beneficial from the detrimental effects of PDE4 inhibitors. These include restricting penetration of the inhibitor into the central nervous system (CNS) (Aoki et al. 2001; Bureau et al. 2006), selectively targeting among the four PDE4 isoenzyme families (Giembycz 2008; Srivani et al. 2008), the generation of “soft” inhibitors (Zhang et al. 2010) as originally defined by Bodor (1984), and, more recently, the development of allosteric inhibitors, which are predicted to have a lower emetic liability (Burgin et al. 2010). In an attempt to identify the molecular targets that are responsible for adverse effects, Merck Frosst synthesized highly emetic, quinoline-based PDE4 inhibitors that covalently label their molecular targets following photoactivation (MacDonald et al. 2000). However, even with this elegant approach, compounds with an ideal pharmacophore have not, thus far, been reported. In this chapter, alternative means of harnessing the clinical efficacy of PDE4 inhibitors for inflammatory lung diseases are described. These concepts are based on the assumption that additive or, ideally, synergistic anti-inflammatory effects can be realized with inhibitors of two or more PDE families or with a PDE4 inhibitor in combination with another anti-inflammatory drug. The overriding premise of this approach is that the therapeutic effects of a PDE4 inhibitor could be realized at a dose lower than would normally be required if it is used as a monotherapy. A corollary to this hypothesis is that the PDE4 inhibitor should also have a much-improved adverse-effect profile.

2 Inhibition of Multiple Phosphodiesterases

Methods to target multiple PDEs include deploying inhibitors that have a pharmacophore that blocks the catalytic site of more single PDE isoenzyme family or by combining two selective inhibitors in a single delivery device, such as an inhaler (Giembycz 2005b). Of the 11 PDE families that have been unequivocally identified, compounds that inhibit PDE4 as well as PDE1, PDE3, PDE5, and/or PDE7 could offer potential opportunities to enhance clinical efficacy. Indeed, additive and/or synergistic effects are produced in a variety of model systems when multiple PDEs are inhibited concurrently. In the context of treating inflammatory airway diseases, these interactions are described below in Sects. 2.1–2.5. For the purposes of this chapter, no distinction is made between the effects of a compound that inhibits two or more PDEs and two isoenzyme-selective inhibitors that are used in combination.

2.1 Inhibition of PDE1 and PDE4

Airway remodeling is a characteristic feature of asthma and refers to several distinct processes that together increase the volume of the respiratory tract wall. In some individuals, airway remodeling leads to an irreversible or *refractory* form of the disease where lung function and symptoms continue to decline despite maximal therapy (Lazaar and Panettieri 2003; Wenzel 2003). On histological examination, remodeling is manifest by mucous gland hyperplasia, subepithelial deposition of collagens, an increase in smooth muscle mass, and revascularization of the mucosa (Jeffery 2001). With respect to smooth muscle, recent investigations have extended the original histopathological findings by demonstrating that both hypertrophy (an increase in cell size) and hyperplasia (an increase in cell number) may contribute to the increase in volume of the airway wall occupied by smooth muscle in fatal *and* nonfatal asthma (Benayoun et al. 2003; Ebina et al. 1993; Heard and Hossain 1973; Hirst et al. 2004; Woodruff et al. 2004). Airway hyperresponsiveness (AHR) is a phenomenon characterized by exaggerated narrowing of the airways in response to a diverse range of (nonspecific) constrictor stimuli. Credible evidence exists that thickening of the airway wall is a major determinant of AHR (Hogg 1996; Martin et al. 2000; Stewart 2001; Wiggs et al. 1990).

Substantial airway remodeling *and* AHR are also characteristic features of COPD (Aoshiba and Nagai 2004; Hogg 2004; Hogg et al. 2004; Hossain and Heard 1970; Jeffery 2001). Indeed, like in asthma, the increase in the volume of the walls of airways is thought to be a principal determinant of AHR (Postma and Kerstjens 1998; Wiggs et al. 1990). To a large degree, remodeling in COPD involves connective tissue deposition in the subepithelial and adventitial compartments (Dunnill et al. 1969; Hogg et al. 2004), but there is also an increase in *smooth*

muscle mass that is most prevalent in the bronchioles (Aoshiba and Nagai 2004; Hogg et al. 2004; Hossain and Heard 1970; Jeffery 2001). This is an important finding as the major site of obstruction in COPD occurs in the smaller airways (<2 mm in diameter) (Hogg et al. 2004).

The possibility that remodeling of the airways may contribute to irreversible airway obstruction and AHR prompted the pharmaceutical industry to look for compounds that arrest, or ideally reverse, this process. With respect to the smooth muscle component, a drug that could prevent mitogenesis could be a therapeutic advance. In this respect, there is precedent that inhibition of PDE1 could be a viable target. Evidence for this possibility derives from studies performed using the human lymphoblastoid RPMI-8392 B-cell line. These cells were established from a patient with acute lymphocytic leukemia and express two major PDEs: PDE1 (probably PDE1B) and PDE4 (Epstein et al. 1987; Jiang et al. 1996). In contrast, normal quiescent human B-lymphocytes lack measurable PDE1 (Epstein et al. 1987; Gantner et al. 1998). Following exposure of normal B-lymphocytes to the mitogen, phytohemagglutinin (PHA), significant PDE1 activity is detected together with mRNA transcripts for PDE1B (Hurwitz et al. 1990; Jiang et al. 1996). The induced enzyme elutes from anion-exchange columns as a 63-kDa protein that is indistinguishable from PDE1B purified from bovine brain (Bentley et al. 1992; Hurwitz et al. 1990). Mitogens are known to promote the transcription of *PDE1B* (Spence et al. 1995, 1997), and it is possible that gene induction occurs when lymphocytes switch from a quiescent to a proliferative phenotype. As cGMP suppresses lymphocyte proliferation (Fischer et al. 2001), the induction of *PDE1B*, which preferentially hydrolyzes cGMP, by PHA could, by lowering the basal cGMP content, represent a physiological response to permit B-cell mitogenesis.

Evidence that PDE1 is implicated in human vascular smooth muscle proliferation is also available (Rybalkin et al. 1997, 2002), and it is not unreasonable to speculate that one or more PDE1 isoenzymes could subservise the same function in airway myocytes. Rybalkin and colleagues found that of the three genes that encode PDE1 isoforms (Bender and Beavo 2006), PDE1C, which hydrolyzes cAMP and cGMP with similar kinetics, was markedly induced in proliferating but not quiescent smooth muscle cells derived from human aorta. Like airway myocytes (Giembycz and Barnes 1991; Torphy et al. 1991, 1993), this enzyme is the predominant PDE in these cells (Rybalkin et al. 1997). Subsequently, it was established that induction of PDE1C correlated with cell cycle progression and that inhibition of this enzyme with either 8-methoxymethyl-3-isobutyl-1-methylxanthine (a modestly selective PDE1 inhibitor) or antisense oligonucleotides directed against *PDE1C* significantly reduced mitogenesis (Rybalkin et al. 2002). As neither zaprinast nor sildenafil (selective PDE5 inhibitors) was anti-mitogenic in the same system, it was concluded that proliferation, due to PDE1C induction, involved increased degradation of cAMP rather than cGMP (Rybalkin et al. 2002). Collectively, these data indicate that induction of PDE1C lowers cAMP in vascular myocytes and so relieves an endogenous “brake” allowing mitogenesis to proceed unhindered. Given that airway remodeling is characteristic of asthma and COPD (Aoshiba and Nagai 2004; Jeffery 2001), dual-selective

inhibitors of PDE1 and PDE4 may work in concert to decrease proliferative processes in airway smooth muscle cells, and so retard the remodeling process while, at the same time, arrest inflammation (via PDE4 inhibition). Indeed, the imidazonaphthyridine derivative (**1**; KF19514; Fig. 1), which selectively inhibits PDE1 and PDE4 with IC_{50} values of 270 and 400 nM, respectively (Manabe et al. 1997), was recently shown to suppress inflammation and airway remodeling in a murine model of chronic asthma (Kita et al. 2009), which provides further evidence that PDE1 may play a role in regulating airway smooth muscle proliferation. In addition, extensive remodeling of the pulmonary vasculature is also prevalent in COPD (see below and Chaouat et al. 2008; Naeije and Barbera 2001). Accordingly, if PDE1 also plays a central role in regulating the proliferation of pulmonary vascular myocytes, then inhibition of this enzyme could also

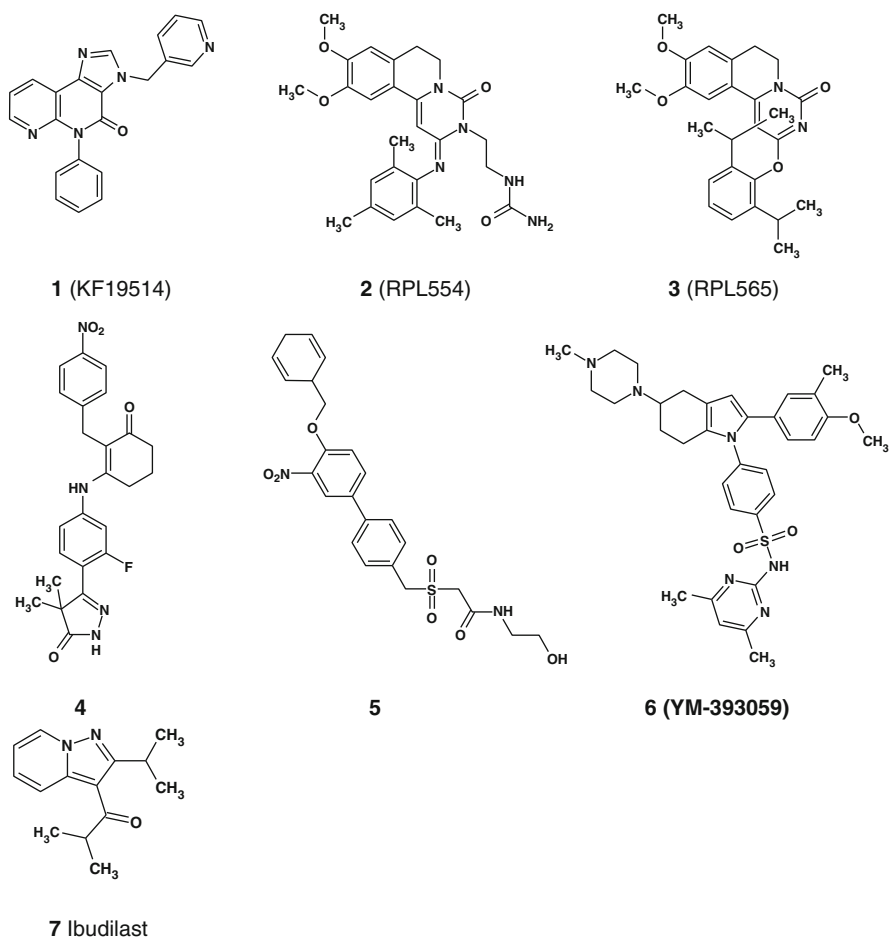


Fig. 1 Dual-selective and nonselective PDE inhibitors

lead to a reduction in pulmonary vascular resistance (see Sect. 2.2). In this respect, it is significant that PDE1A and PDE1C are upregulated in “remodeled” pulmonary artery vessels harvested from patients with idiopathic pulmonary hypertension (PH) (Schermuly et al. 2007).

2.2 Inhibition of PDE3 and PDE4

Interest in PDE3 as a target for the treatment of asthma and COPD originated from the finding that selective PDE3 inhibitors promote bronchodilation in humans (Bardin et al. 1998; Fujimura et al. 1995; Leeman et al. 1987; Myou et al. 1999). Thus, mechanistically, inhibitors that block both PDE3 and PDE4 should improve lung function, by promoting airway smooth muscle relaxation, and suppress inflammation, respectively. Accordingly, such drugs are predicted to have superior clinical efficacy over compounds that selectively block PDE4. Furthermore, a potentially more significant effect is that PDE3 may also have clinically relevant actions on certain proinflammatory and immune cells especially during concurrent PDE4 inhibition. For example, T-lymphocytes, macrophages, monocytes, epithelial and endothelial cells, dendritic cells, and airway myocytes coexpress PDE3 and PDE4 (Banner and Press 2009; Torphy 1998). In vitro studies have shown that while PDE3 inhibitors generally have little or no effect on T-cell proliferation or on IL-2 generation, they significantly enhance the repressive effect of a PDE4 inhibitor (Giembycz et al. 1996; Robicsek et al. 1991). Similar data have been reported for the inhibition of proinflammatory responses in human alveolar macrophages (Schudt et al. 1995), monocyte-derived dendritic cells (Gantner et al. 1999), airway epithelial cells (Wright et al. 1998), human lung fibroblasts (Selige et al. 2010), and human lung microvascular endothelial cells (Blease et al. 1998).

Several dual-selective inhibitors of PDE3 and PDE4 have been developed and evaluated in humans including zardaverine, ben(z)afentrine, tolafentrine, and pumafentrine (see Chap. 6.2 and Banner and Press 2009). Since the early 2000s, there has been a dearth of peer-reviewed data from which to gauge the clinical progress of these molecules and one must assume that the development of many of those originally described compounds has been discontinued. However, the development of new PDE3/PDE4 candidates continues, suggesting that early testing in humans has begun or will likely follow in the short term. For example, the pharmacology of two long-acting pyrimido[6,1-*a*]isoquinolin-4-ones, which are derivatives of trequensin and are protected by a patent from Verona Pharma (Oxford and Jack 2000), have been described (Boswell-Smith et al. 2006). Compound **2** (RPL 554; Fig. 1) is a very potent PDE3 inhibitor ($IC_{50} = 400$ pM) with an IC_{50} (1.5 μ M) that is >3,000-fold lower for inhibition of PDE4 ($IC_{50} = 1.5$ μ M). Currently, this compound is in Phase I/IIa studies for allergic rhinitis and asthma (Pages et al. 2009). Given the isoenzyme selectivity of this compound, it seems likely that the dominant activity of **2** will be PDE3 inhibition, which might limit

clinical efficacy due to potential cardiovascular toxicity (see below). Nevertheless, in September 2009, Verona Pharma announced that trials of **2** had been successfully completed. According to the company's website, the compound has a "good safety profile and had beneficial effects in terms of bronchodilation and bronchoprotection in asthmatics and a reduction in the numbers of inflammatory cells in the nasal passages of allergic rhinitis patients" (see <http://www.veronapharma.com/s/LeadDrugRPL554.asp>). Compound **3** (RPL 565; Fig. 1) lacks the urea side chain of **2** and has an ether link to a dipropan-2-ylbenzene moiety. Unlike **2**, this derivative inhibits PDE3 and PDE4 with similar potencies ($IC_{50} = 107$ nM and 1.2 μ M, respectively) (Boswell-Smith et al. 2006). Although it might be expected that **3** would be the desired development candidate because of its ability to inhibit PDE3 and PDE4 in vivo and so maximize the chances of synergy, no clinical data have been reported.

Several other companies including Nycomed, Kyorin, and the Leiden/Amsterdam Centre for Drug Research have patents protecting dual PDE3/PDE4 inhibitors of varied structural classes. Interested readers should consult Pages et al. (2009) for details.

While the scientific rationale is clear for inhibiting, simultaneously, both PDE3 and PDE4 (see above), there may be major safety issues with this approach. Specifically, concerns about cardiovascular toxicity may explain why there are relatively few patents claiming dual-selective compounds for respiratory diseases when compared to highly selective inhibitors of PDE4. Historically, PDE3 inhibitors were developed as "safer" alternatives to cardiac glycosides for the treatment of dilated cardiomyopathy. Although this objective was realized in acute dosing studies with notable beneficial effects on myocardial contractility and on vascular smooth muscle tone, chronic treatment resulted, paradoxically, in a significant increase in mortality (Amsallem et al. 2005; Movsesian 2003). These findings are of concern as some individuals who suffer with COPD also have right heart failure secondary to PH (Naeije 2003). Thus, a PDE3/PDE4 inhibitor would, presumably, be contraindicated in this clinical setting. Moreover, the long-term effect of this type of drug in individuals with mild COPD and normal lung function is unknown.

Another potential cause for concern is arteritis, which is believed to be due to changes in hemodynamics produced by prolonged vasodilation of various vascular beds. There is an extensive literature on PDE3 inhibitor-induced arteriopathy in laboratory animals with the splanchnic vessels and coronary arteries of rats and dogs, respectively, being the most susceptible to lesions (Joseph 2000). Whether arteriopathies evoked by PDE3/PDE4 inhibitors are to be expected in humans with chronic use, as would be required in COPD, is unknown. However, the nonselective PDE inhibitors theophylline, pentoxifylline, and ibudilast, and the selective PDE3 inhibitor, cilostamide, have been used clinically for many years with no evidence, to date, of vascular toxicity.

If PDE3/PDE4 inhibitors are to be an effective treatment option, a clinically meaningful bronchodilator response would be required without coincident changes in cardiac function and systemic blood pressure (Brunnee et al. 1992; Wilmshurst and Webb-Peploe 1983; Yamashita et al. 1990). The fact that pumafentrine

progressed to phase II clinical trials may suggest that systemic exposure and/or cardiovascular toxicity of PDE3/PDE4 inhibitors can be minimized. Indeed, because of synergy in target cells, it is logical to predict that proportionally lower doses of dual-selective inhibitors will be required to elicit the same degree of therapeutic benefit relative to a PDE4 inhibitor alone as a monotherapy. Selectively targeting the respiratory tree through formulation and/or route of administration are validated means of gaining tissue-specific drug delivery. In addition, exploiting the fact that in PDE3 isoforms are encoded by two genes (PDE3A and PDE3B; Bender and Beavo 2006) could also offer a more novel approach to reduce cardiovascular adverse events (Thompson et al. 2007). In this respect, baseline cardiovascular function (blood pressure, heart rate (HR), left ventricular pressure (LVP), and cardiac contractility) and adenosine diphosphate- and collagen-induced platelet aggregation *in vitro* were unaffected in *pde3b*-deficient mice when compared to wild-type animals. In contrast, mice lacking *pde3a* had an elevated HR and LVP was significantly reduced (Sun et al. 2007). Moreover, the PDE3 inhibitor, cilostamide, did not change any of the aforementioned hemodynamic parameters in *pde3a* knockout mice, whereas in wild-type mice and animals deficient in *pde3b*, HR and cardiac contractility and BP and LVP were significantly increased and decreased, respectively (Sun et al. 2007). Clearly, these data show that, in mice, PDE3A is the major regulator of several cardiovascular parameters. What is unknown are the PDE3 isoenzymes that, when inhibited, promote bronchodilation and enhance the anti-inflammatory effect of PDE4 inhibitors. Indeed, human airway smooth muscle cells express mRNA for both PDE3 isoforms (authors' unpublished observations) and the expression of these variants across proinflammatory and immune cells has not been cataloged. Nevertheless, subtype-selective compounds have been described including **4** and **5** (Fig. 1) from Merck and ICOS, respectively, that are >30-fold selective for the PDE3B isoform (Edmondson et al. 2003; Snyder et al. 2002). Thus, tools are beginning to emerge with which to tease out, pharmacologically, the functional effects mediated by inhibition of PDE3A and PDE3B.

2.3 Inhibition of PDE4 and PDE5

Chronic generalized alveolar hypoxia occurs in diseases associated with decreased ventilation including COPD. Accordingly, many patients with COPD have coexisting PH, although the exact prevalence is unclear (see Jyothula and Safdar 2009). This condition is thought to be multifactorial due to complex interactions involving hypoxic pulmonary vasoconstriction (HPV), pulmonary and systemic inflammation, a loss of pulmonary capillaries, and, potentially, hyperinflation, which may cause mechanical injury (reviewed in Chaouat et al. 2008; Jyothula and Safdar 2009). Although the increase in pulmonary artery pressure (Ppa) is usually mild to moderate, some individuals suffer from right heart failure secondary to severe PH. In addition, there can be extensive remodeling of the pulmonary vasculature in

COPD with all layers of pulmonary vessels (intima, media, and adventitia) being affected (Timms et al. 1985).

There is persuasive clinical evidence that PDE5 inhibition could arrest, or even reverse, these pathological effects of alveolar hypoxia. Thus, both short- and long-term dosing trials have clearly shown that inhibitors of PDE5 decrease pulmonary vascular resistance (PVR) and/or Ppa in humans with PH (Bharani et al. 2003; Chockalingam et al. 2005; Galie et al. 2005; Ghofrani et al. 2004; Lepore et al. 2005; Michelakis et al. 2002, 2003; Prasad et al. 2000; Sastry et al. 2002, 2004; Steiner et al. 2005; Wilkens et al. 2001). Therefore, it is not unreasonable to suspect that this class of drug could also be useful in those patients that have COPD-associated PH. In a small study involving five patients with COPD with coexisting PH at rest, oral sildenafil (50 mg) attenuated the increase in mean Ppa during submaximal exercise in the absence of any change in stroke volume or cardiac output (Holverda et al. 2008). In a similar study involving six patients with severe COPD and elevated Ppa at rest, oral sildenafil (50 mg b.i.d. for 3 months) significantly reduced PVR and mean Ppa. An improvement in exercise tolerance using the 6 min walk test was also reported (Alp et al. 2006). Similar data have been described in another small study involving seven patients with severe COPD (GOLD Stage 4) (Madden et al. 2006). While these data are encouraging, the obvious limitation is the low sample size. Accordingly, these results need to be confirmed in much larger populations of patients with COPD-related PH.

In addition to relaxing pulmonary vascular smooth muscle, Charan (2001) has published case reports describing beneficial effects of sildenafil on lung function. In two patients with erectile dysfunction who had concurrent COPD, oral sildenafil produced a rapid (within 1 h of administration) and long-lasting improvement in FEV₁ and forced vital capacity (24 and 12%, respectively) indicative of direct bronchodilation. Finally, there is *in vitro* evidence that PDE5 inhibitors prevent human platelet aggregation (Yu et al. 1996), which is prevalent in COPD (Cordova et al. 1985) and also block the proliferation of myocytes derived from the human pulmonary artery (Wharton et al. 2005). Thus, PDE5 inhibitors can, in theory, exact multiple therapeutically beneficial effects on COPD-associated pulmonary vascular pathologies. Clearly, the combination of such a drug with a PDE4 inhibitor, which may also be antiproliferative in human pulmonary vascular myocytes (Growcott et al. 2006), could offer a superior medicine to either drug alone since both the inflammatory and vascular components of COPD would be targeted.

2.4 Inhibition of PDE4 and PDE7

All human immune and proinflammatory cells that have been studied express mRNA and protein for PDE7A (Smith et al. 2003). The expression of this enzyme family is, therefore, very similar to the distribution of PDE4. CD4⁺ and CD8⁺ T-lymphocytes express relatively high levels of PDE7A1 that are readily detected by western blotting. Human airway smooth muscle cells, blood monocytes, and

lung macrophages together with several cell lines including HUT-78 (T-cell) and BEAS-2B (epithelial) are also PDE7A⁺ (Secchiero et al. 2000; Smith et al. 2003). In contrast, PDE7A expression is seemingly much lower in human neutrophils since it was not identified by western blotting, although the protein was clearly labeled using immunoconfocal laser microscopy (Smith et al. 2003). This more sensitive technique has also been employed to determine the expression profile of PDE7A in cells present in sputum and bronchoalveolar lavage fluid in which neutrophils from both normal subjects and individuals with asthma and COPD were clearly labeled (Smith et al. 2003).

The coincident expression of PDE7 and PDE4 in many cells relevant to the pathogenesis of chronic airway inflammation nourished the idea that selective PDE7A inhibitors could be anti-inflammatory with an improved adverse-effect profile of PDE4 inhibitors (Giembycz and Smith 2006a, b). However, despite the discovery of PDE7 in the early 1990s (Michaeli et al. 1993), there were until 2001 surprisingly few reports of selective inhibitors. However, many examples of compounds with PDE7 inhibitory activity have now been described (reviewed in Giembycz and Smith 2006a, b; Gil et al. 2008; Vergne et al. 2005) and some of these have been evaluated in preclinical models of inflammation including BRL 50481 (Smith et al. 2004), PF 0332040 (Jones et al. 2006), ABS16165 (Goto et al. 2009; Kadoshima-Yamaoka et al. 2009a, b, c), T-2585 (Nakata et al. 2002), and BMS 586353 (Yang et al. 2003). What has emerged thus far from these investigations is controversy. Thus, PDE7 inhibitors have been reported to be active and inactive in suppressing indices of inflammation *in vitro* and *in vivo*. Nevertheless, of potential interest is that although the PDE7A inhibitor, BRL 50481, did not attenuate the proliferation of human T-cells *per se*, it significantly augmented the antimitogenic and cAMP-elevating activity of the PDE4 inhibitor, rolipram (Smith et al. 2004). Similarly, the suppression by PDE4 inhibitors of tumor necrosis factor- α (TNF- α) release from LPS-stimulated human monocytes and lung macrophages was significantly enhanced by BRL 50481 (Smith et al. 2004). Collectively, these data are reminiscent of the behavior of PDE3 inhibitors in human T-cells (see Sect. 2.2) and tempt speculation that additive or even synergistic anti-inflammatory effects could be realized with a dual PDE4/PDE7 inhibitor over a PDE4 inhibitor alone (Giembycz 2005a; Vijayakrishnan et al. 2007). Moreover, it is also possible that this dual PDE inhibition approach would increase tolerability and avoid the potential cardiovascular complications that are of concern with dual PDE3/PDE4 inhibitors (described in Sect. 2.2).

Despite the potential therapeutic utility of PDE4/PDE7 inhibitors, very few studies have investigated the biology of these compounds and whether they are, in fact, superior to selective PDE4 inhibitors in suppressing indices of inflammation. Only compound **6** (YM-393059; Fig. 1), which inhibits PDE4 and PDE7 with IC₅₀ values of 630 and 14 nM, respectively (Yamamoto et al. 2006b), has demonstrated efficacy in preclinical models of inflammation with a reduced emetic liability (Yamamoto et al. 2006a, b; Yamamoto et al. 2007a). However, it is difficult to determine the extent to which inhibition of PDE7 contributed to these desirable outcomes.

2.5 Nonselective PDE Inhibition

A logical extension to the aforementioned discussion on dual-selective inhibitors is to consider whether nonselective compounds would have even greater efficacy in combating chronic airway inflammation. It is well reported that theophylline may exert anti-inflammatory activity in human subjects with asthma when given at sub-bronchodilator doses where the plasma concentration is between 5 and 10 $\mu\text{g/ml}$. Although this beneficial effect is often attributed to mechanisms unrelated to PDE inhibition, this interpretation is unnecessary. Indeed, theophylline even at a concentration of 5 $\mu\text{g/ml}$ (27 μM), and taking into account plasma protein binding, will inhibit, albeit modestly, PDEs 1–5 by up to $\sim 20\%$ depending on the isoenzyme. Thus, in addition to any beneficial effects produced by the inhibition of each PDE in isolation (see Sects. 2.1–2.4), the probability for functional additivity and, indeed, synergy when multiple PDEs are inhibited concurrently in the same cell types cannot be overstated (Fig. 2) (Giembycz 2005b). Accordingly, it is not unreasonable to believe that second-generation, nonselective PDE inhibitors could exhibit superior clinical efficacy over theophylline or compounds that selectively target PDE4. Indeed, simultaneous inhibition of multiple PDEs should exert clinically relevant effects on multiple proinflammatory processes including airway remodeling (PDE1-regulated), endothelial cell permeability (PDE2-regulated), mast cell stabilization (PDE3-mediated), and remodeling of the pulmonary vasculature (PDE5-regulated).

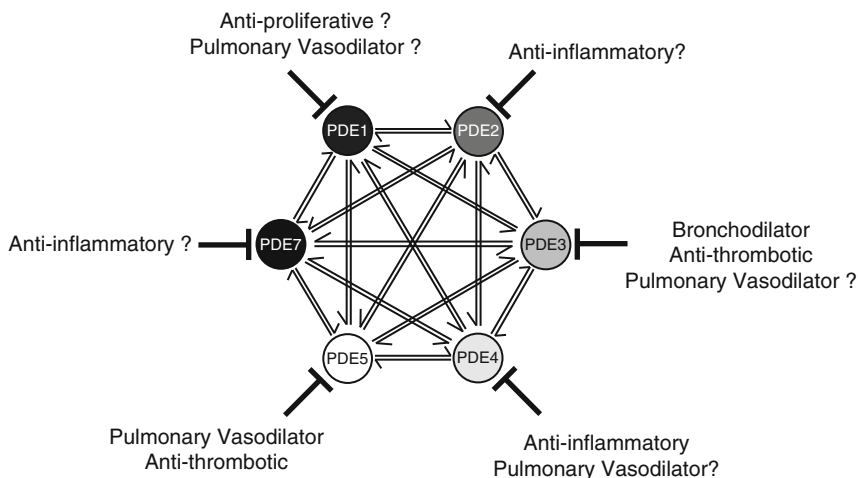


Fig. 2 Functional consequences of selective phosphodiesterase (PDE) inhibition in relation to the treatment of COPD. The cartoon shows clinically beneficial effects that may be produced following the selective inhibition of PDEs 1, 2, 3, 4, 5, and 7. Additive and/or synergistic effects may be produced if two or more PDEs are inhibited concurrently (*reversible arrows*). For example, it is well established that a PDE3 inhibitor and a PDE7 inhibitor will potentiate the effect of a PDE4 inhibitor. Similar interactions have been reported for other PDEs. Accordingly, a nonselective PDE inhibitor is predicted to have a beneficial clinical impact on multiple facets of the disease process and may be a far superior drug that inhibits PDE4 selectively

Beneficial actions on airway smooth muscle (PDE3-mediated) and pulmonary vascular tone (PDE4/PDE5-mediated) are also predicted (Fig. 2). Moreover, because the mechanism of action relies on a modest inhibition of multiple PDEs, the potential for emesis and related gastrointestinal adverse events, which are PDE4-mediated, could be minimized. Clearly, the design of such compounds should probably avoid the xanthine nucleus as a starting template to avoid potential complications associated with the antagonism of adenosine receptors, which mediate many of the adverse cardiovascular and CNS effects of theophylline (Barnes 2003).

It is worth noting that the pyrazolo-pyridine **7**, ibudilast (marketed variably as *Ketas*[®], *Pinatos*[®], and *Eyevinat*[®]; Fig. 1), is a relatively nonselective PDE inhibitor (Gibson et al. 2006; Souness et al. 1994) and has been available in Japan for >20 years for several indications including asthma (Rolan et al. 2009). The compound has demonstrated bronchodilator, vasodilator, and in vitro anti-inflammatory activity. In humans, it displays superior pharmacokinetics, is better tolerated, and, accordingly, has an improved adverse-effect profile over theophylline (Rolan et al. 2009). On the basis of these data, it is tempting to speculate that the clinical effects of ibudilast in asthma are due to the inhibition of multiple PDEs. However, there are very few appropriately designed and powered studies that have shown clinical efficacy (Rolan et al. 2009). Consequently, it is difficult to know whether the recommended oral dose of ibudilast (10 or 20 mg b.i.d.) achieves a concentration in the lung that is necessary for anti-inflammatory activity, due to PDE inhibition, to be realized.

3 Combination Therapies Involving Phosphodiesterase 4 Inhibitors: The Obvious and the Novel

An alternative approach to harness the clinical efficacy of PDE4 inhibitors is to use them in combination with other bronchodilator and anti-inflammatory drugs to achieve additive or synergistic effects. Several logical drug partners include long-acting β_2 -adrenoceptor agonists (LABAs), inhaled corticosteroids (ICSs; more correctly known as glucocorticoids), and, perhaps, agonists of nuclear hormone receptors other than the glucocorticoid receptor (GR). The theoretical mechanisms of action of these combination therapies and their relative merits are discussed below.

3.1 PDE4 Inhibitor/ β_2 -Adrenoceptor Agonist Combination Therapies

In the preceding sections, the potential clinical benefit of using inhibitors that target more than a single PDE family in the treatment of chronic airway inflammation is described. However, in addition to any disease-modifying characteristics that such compounds might have, it is likely that inhibition of PDE4 may also prove to be an effective strategy for increasing the therapeutic effect of existing agents.

This could be particularly useful in COPD where ICSs, which are the gold standard for treating the inflammation in asthma, are relatively ineffective (cf. asthma). In this respect, patients with mild disease are often treated with LABAs and short-acting β_2 -adrenoceptor agonists (SABAs) on an as-needed basis (O'Donnell et al. 2008). Given that these drugs act principally via the cAMP/cAMP-dependent protein kinase (PKA) signaling pathway (Giembycz and Newton 2006), it is not unreasonable to suggest that the presence of a PDE4 inhibitor may enhance β_2 -adrenoceptor-mediated responses. However, it is generally believed that *pde4d* inhibitors do not effect bronchodilation in humans (Grootendorst et al. 2003) so improvements in lung function due to increased smooth muscle relaxation are unlikely. Nevertheless, although SABAs and LABAs do not suppress inflammation in human subjects with asthma or COPD when administered as monotherapies (Howarth et al. 2000; Roberts et al. 1999; Sindi et al. 2009), it is entirely possible that by enhancing cAMP synthesis in target cells they may boost the anti-inflammatory actions of a PDE4 inhibitor. In fibroblasts from *pde4d*-deficient mice, cAMP accumulation and cAMP-response element-binding protein phosphorylation were markedly enhanced following the addition of a β_2 -adrenoceptor agonist when compared to fibroblasts from wild-type animals (Bruss et al. 2008). Moreover, in monocytes, the SABAs, salbutamol, and procaterol, interact synergistically with rolipram on cAMP accumulation and in the activation of PKA (Seldon et al. 1995). Similar data have also been reported for human neutrophils (Meliton et al. 2006) and human eosinophils (Ezeamuzie 2001; Meliton et al. 2003). However, in proinflammatory and immune cells the balance of evidence indicates, paradoxically, that despite synergy at the level of cAMP accumulation and downstream signal amplification, PDE4 inhibitors and β_2 -adrenoceptor agonists in combination usually act additively in the suppression of a variety of proinflammatory mediators such as TNF- α (Seldon et al. 1995). Thus, given that the first PDE4 inhibitor, roflumilast, has now been approved for the treatment of severe COPD, it is not unreasonable to predict that in certain patient populations this class of drugs will provide additional benefit as an add-on therapy when symptoms are not adequately controlled by a LABA alone (Gross et al. 2010). Indeed, this suggestion is supported by the results of a recent 12-month phase III clinical trial in patients with severe COPD where postbronchodilator FEV₁ at the end of the study was 60 ml greater in patients treated with the combination of roflumilast and the LABA, salmeterol, when compared to patients treated with salmeterol alone (Calverley et al. 2009).

3.2 PDE4 Inhibitor/Glucocorticoid Combination Therapies

3.2.1 Clinical Observations

Another attractive option to optimize anti-inflammatory activity is to combine a PDE4 inhibitor with an ICS. In asthma, glucocorticoids downregulate the expression of proinflammatory genes and, thereby, dampen down inflammation and

reduce symptoms (Barnes 2006). In addition, large multicenter clinical trials have clearly demonstrated that the clinical efficacy of an ICS can be enhanced by a LABA (Shrewsbury et al. 2000). Given the lack of a clinically significant anti-inflammatory effect of LABAs in asthma when given as a monotherapy, it seems likely that some form of synergy must occur between these two classes of drug to account for their superior efficacy when they are used in combination (Giembycz et al. 2008). Similarly, the balance of evidence suggests that *Advair*[®], a LABA/ICS combination therapy, may produce similar, albeit less pronounced, beneficial effects in patients with COPD (Celli et al. 2008; Jenkins et al. 2009). While the detailed biochemical mechanisms that lead to these clinical observations remain unclear (but see Sect. 3.2.2), it is reasonable to believe that other drugs that raise intracellular cAMP, such as PDE4 inhibitors, may also enhance the clinical efficacy of glucocorticoids (Newton et al. 2010) in addition to exerting anti-inflammatory activity in their own right. Support for this assumption comes from clinical studies with the nonselective PDE inhibitor, theophylline. For example, in subjects with moderate asthma, low-dose, inhaled budesonide together with theophylline (mean plasma concentration = 8.7 µg/ml) and high-dose inhaled budesonide as a monotherapy produced similar clinical benefits in lung function, severity of disease, and variability in peak expiratory flow, which is a correlate of AHR (Evans et al. 1997). This general finding has been independently confirmed in several other trials in which the interaction of glucocorticoids and theophylline in the control of asthma has been studied (Lim et al. 2000; Ukena et al. 1997).

Clinical reports from the arthritis literature also support the general idea that PDE inhibitors may augment glucocorticoid action. One of these described the results of a phase IIa trial in which low-dose prednisolone in combination with dipyridamole, a nonselective PDE inhibitor and adenosine uptake inhibitor, was compared against placebo in subjects with hand osteoarthritis (Kvien et al. 2008). The primary efficacy end point measure was change in the Australian/Canadian visual analog pain scale, and this combination therapy, named *Synavive*[®] (CRx-102), significantly reduced pain relative to placebo (Kvien et al. 2008). Whether this benefit is due to a synergistic interaction between the glucocorticoid and the ability of dipyridamole to nonselectively inhibit PDEs was not explored. Nevertheless, this is an intriguing possibility and similar investigations have now been conducted in COPD and rhinitis with selective PDE4 inhibitors. Thus, in a COPD study, Calverley et al. (2007) reported that roflumilast produced a sustained, albeit modest, improvement in postbronchodilator FEV₁ in subjects with severe disease (GOLD stages III and IV) in whom the use of ICS was permitted (Calverley et al. 2007). Thus, there was an enhanced clinical benefit in lung function over that produced by the ICS alone. Moreover, in May 2008, GlaxoSmithKline completed a phase II study in which the effect of intranasal fluticasone, alone and in combination with an extraordinary potent PDE4 inhibitor, GSK 256066 (Woodrow et al. 2009), was examined in a cohort of subjects with seasonal allergic rhinitis (<http://clinicaltrials.gov/ct2/show/NCT00612820>). The results of that study are awaited with interest.

3.2.2 How Glucocorticoids Work

To understand how PDE4 inhibitors and glucocorticoids might interact to deliver superior clinical benefit, an appreciation of the mechanisms of action of glucocorticoids is required. In asthmatic subjects, *in vivo*, glucocorticoids produce a profound reduction in pulmonary leukocyte burden that is associated with chronic inflammation (Barnes 2006). In particular, glucocorticoids dramatically reduce eosinophil numbers in the lung by inhibiting the expression of numerous proinflammatory mediators, including cytokines, chemokines, inflammatory proteins, and their receptors, as well as adhesion molecules. The primary mechanism of this anti-inflammatory action of glucocorticoids is the repression of proinflammatory gene expression (Newton 2000).

Mechanistically, glucocorticoids act via the GR (NR3C1), which on binding glucocorticoid translocates from the cytoplasm to the nucleus, where it interacts with DNA sequences in the promoters of target genes to modulate their transcriptional activity (Pratt et al. 2004). These regulatory sites were initially described as palindromic sequences referred to as simple glucocorticoid response elements (GREs), to which the agonist-bound GR interacted as a dimer (Dahlman-Wright et al. 1991). However, it is now clear that the GR can interact with multiple transcription factors to promote transcription via a multitude of discrete interactions (Newton and Holden 2007). Collectively, this ability to induce gene transcription is known as *transactivation* and this was previously perceived as being responsible for many of the adverse effects, often metabolic, that manifest following longer term, high-dose glucocorticoid therapies (Schacke et al. 2002). However, ongoing characterization of the human genome has led to the realization that many hundreds of genes are induced by glucocorticoids and many of these have, potentially, profound anti-inflammatory activity (see Newton and Holden 2007). Furthermore, many investigators report that the ability of glucocorticoids to repress proinflammatory gene expression is abrogated under conditions of translational or transcriptional blockade, thereby implying that these repressive effects are themselves dependent on gene expression (Newton and Holden 2007). Thus, genes such as glucocorticoid-induced leucine zipper (*GILZ*), mitogen-activated protein kinase phosphatase (*MKP*) 1, and tristetraprolin are all induced by glucocorticoids and may, variously, repress proinflammatory gene expression at transcriptional, posttranscriptional, translational, or even posttranslational levels (Newton and Holden 2007). Alternatively, the agonist-bound GR is believed to directly interfere with the induction of key proinflammatory transcription factors such as nuclear factor kappaB (NF- κ B) and activator protein (AP)-1 (De Bosscher et al. 2003). In this model, the GR is believed to recruit repressor molecules, such as histone deacetylase (HDAC) 2, to reduce transcriptional activation (Ito et al. 2000). However, the distinction between direct repression by the GR (often referred to as *transrepression*) and the repression of transcription that is exerted by glucocorticoid-induced genes has become eroded with the findings that glucocorticoid-inducible genes including *GILZ* and *MKP-1* may both play roles in the repression of NF- κ B- and AP-1-dependent transcription (Diefenbacher et al. 2008; Eddleston et al. 2007; King et al. 2009; Mittelstadt and

Ashwell 2001). Furthermore, glucocorticoids are believed to induce the expression of a number of genes that promote cell cycle arrest and apoptosis in various cell types (Amsterdam and Sasson 2002; Reisman and Thompson 1995; Wang et al. 2003). Thus, GR-mediated changes in gene expression can produce many of the wide ranging effects that are normally associated with the anti-inflammatory actions of glucocorticoids (Clark 2007; Newton and Holden 2007; Stellato 2004).

3.2.3 Enhancement of Glucocorticoid Action by cAMP-Elevating Drugs

Given clinical data showing a clear enhancement of glucocorticoid action by LABAs, it should be possible to use *in vitro* models to interrogate forms of interaction. There are considerable data to suggest that β_2 -adrenoceptor agonists acting via the cAMP–PKA pathway may repress NF- κ B-dependent transcription and proinflammatory gene expression (Ammit et al. 2002; Hallsworth et al. 2001; Kaur et al. 2008b; Meja et al. 2004; Ye 2000). While similar repressive effects on cytokine gene expression are reported with PDE4 inhibitors, such as rolipram (Seldon et al. 1995), these effects vary considerably depending on the cell type, stimulus, and experimental conditions. Furthermore, it is equally clear that agents that elevate cAMP can also *enhance* the expression of certain proinflammatory genes including granulocyte-colony-stimulating factor, interleukin (IL)-6, and IL-8 (Ammit et al. 2000, 2002; Clarke et al. 2005a, b; Holden et al. 2010). Moreover, Hertz and colleagues reported in 2009 the results of a microarray study in which forskolin upregulated 334 genes out of a total 8,530 genes in monocyte-derived macrophages (Hertz et al. 2009). Significantly, CC and CXC chemokines were strongly induced (>10-fold) and some, such as CXCL5 (*aka* epithelial-derived neutrophil-activating peptide 78) and CXCL7 (*aka* neutrophil-activating peptide 2), were increased by greater than 50-fold. Similar data were found with rolipram in the presence of PGE₂ (Hertz et al. 2009), indicating that in respiratory diseases, PDE4-mediated cAMP elevation could, in theory, promote the accumulation of CCR2- and CXCR2-sensitive cells to the lung and so, paradoxically, enhance pulmonary inflammation. Thus, an unambiguous argument for anti-inflammatory gain is not forthcoming and this is consistent with clinical data showing that LABAs, which also elevate cAMP, are not anti-inflammatory in asthma (Howarth et al. 2000; Roberts et al. 1999; Sindi et al. 2009). On the basis of these results, it seems likely that a similar outcome may also apply to the anti-inflammatory potential of other cAMP-elevating agents such as PDE4 inhibitors when given as a *monotherapy*. Indeed, although hints of an anti-inflammatory effect of PDE4 inhibitors have been reported in patients with COPD (Gamble et al. 2003; Grootendorst et al. 2007) and more recently in asthma (Singh et al. 2010), the ability of these drugs also to promote the expression of proinflammatory genes may explain why their predicted anti-inflammatory potential has not come to fruition.

Despite the lack of demonstrable anti-inflammatory activity of LABAs as monotherapies (Sindi et al. 2009), the superior clinical activity achieved when a LABA is combined with an ICS is unequivocal when compared to high-dose ICS

alone (Shrewsbury et al. 2000). Thus, the combination therapy provides superior control of asthma and reduces the frequency of exacerbations, indicating that the underlying lung inflammation is reduced (Pauwels et al. 1997). In an effort to understand this phenomenon, many investigators have examined the effect of LABAs and glucocorticoids alone and together on the expression of proinflammatory genes. In almost all cases, the genes selected for study were, typically, suppressed in an additive manner (reviewed in Newton et al. 2010, but see Pang and Knox 2000). Consequently, it seems unlikely that the clinical observations produced by the combination therapy reflect the ability of LABAs (and, by inference, PDE4 inhibitors) to directly modulate proinflammatory gene expression. Alternatively, it could be argued that the effect of combination therapies on the genes that would be induced by a LABA or a PDE4 inhibitor may provide some explanation. Thus, the release of certain cytokines and chemokine from bronchial epithelial cells and monocyte-derived macrophages is significantly enhanced by LABAs and PDE4 inhibitors (Hertz et al. 2009; Holden et al. 2010). However, in the presence of dexamethasone many of these gene induction events will be abolished or greatly diminished to the same or a similar extent when the LABA was absent (Holden et al. 2010). If such an effect is seen for the majority of the proinflammatory genes that are induced by LABAs and PDE4 inhibitors, then these findings could explain, at least in part, how cAMP-elevating drugs and glucocorticoids repress, synergistically, proinflammatory gene expression.

While LABAs may promote the recruitment of HDACs to the promoters of proinflammatory genes (Nie et al. 2005b), there is little evidence that cAMP can enhance the ability of glucocorticoids to repress, or *transrepress*, gene transcription. Consequently, we suggest that such mechanisms may not account for the superior clinical activity of ICS/LABA combination therapies in asthma and COPD (Newton et al. 2010). Conversely, PKA activation has been shown to enhance GRE-dependent transcription in a variety of cells types, including proinflammatory and immune cells (Rangarajan et al. 1992), and this extends to human bronchial epithelial cells and human airway myocytes with SABAs and LABAs (Kaur et al. 2008a). Of prime significance is that “real” glucocorticoid-inducible genes that have anti-inflammatory potential are also upregulated by LABAs providing a plausible mechanistic basis for the clinical superiority of LABA/ICS combination therapies (Giembycz et al. 2008). Further support for this concept is that agonists of the prostacyclin receptor, which typically couples to adenylyl cyclase, similarly enhance glucocorticoid-dependent transcription of several genes (e.g., *GILZ*, *MKP-1*, and *p57^{kip2}*) in bronchial epithelial cells that are predicted to suppress inflammation (Wilson et al. 2009).

In light of the aforementioned discussion, it is possible that a PDE4 inhibitor could also improve lung function by enhancing the ability of ICSs to induce anti-inflammatory genes (Giembycz et al. 2008). Some evidence for this comes from the psychiatry and cancer literature. Thus, rolipram has been shown to augment dexamethasone-induced activation of luciferase reporter genes that were stably transfected into mouse L929 cells and rat C6 glioma cells (Miller et al. 2002).

More recently, this effect on simple GRE-dependent transcription has been confirmed in leukemic cells from patients with B-cell chronic lymphocytic leukemia (Tiwari et al. 2005) and, of relevance to the subject of this chapter, in human bronchial epithelial cells (Kaur et al. 2008a). It is, perhaps, more significant that in human acute T-lymphocytic leukemia cells, certain proapoptotic proteins (p53, p21^{WAF1/CIP1}) are also upregulated by both PDE4 inhibitors and glucocorticoids alone and in combination leading to additive and/or synergistic proapoptotic effects (Ogawa et al. 2002). Thus, if these types of interactions occur in proinflammatory and immune cells implicated in the pathogenesis of diseases such as asthma and COPD, then PDE4 inhibition could enhance the ability of glucocorticoids to induce anti-inflammatory genes. In addition, a potential advantage of this approach is that a low dose of a PDE4 inhibitor, which may be subtherapeutic as a monotherapy, could boost glucocorticoid action in the same way as a LABA. Finally, there are data indicating that in a T-leukemia cell line, such effects are not limited to PDE4, but also extend to inhibitors of PDE3 and PDE7 (Dong et al. 2010), indicating the potential for the development of additional, potentially highly effective, novel combination therapies with which to treat chronic airway inflammation (Zhang and Insel 2004).

In considering the clinical utility and advantages of PDE4 inhibitor/glucocorticoid combination therapies over a PDE4 inhibitor alone as a monotherapy, it is important to appreciate that many of the proinflammatory genes that may be induced by cAMP (Ammit et al. 2000; Hertz et al. 2009; Holden et al. 2010) are likely to be repressed by glucocorticoids. Thus, a PDE4 inhibitor and an ICS given together may be a considerably superior medicine for several reasons. First, the cAMP signal generated in response to PDE4 inhibition may enhance the maximal transcriptional activity of the glucocorticoid. Second, the PDE4 inhibitor will exert anti-inflammatory activity independently of its effect on GR-mediated *transactivation* of anti-inflammatory genes. Third, the undesirable ability of PDE4 inhibitors to induce proinflammatory genes will be negated by the glucocorticoid.

An appreciation of rate of cAMP synthesis in target tissues is also a critical factor that will influence the efficacy of a PDE4 inhibitor. If adenylyl cyclase activity in those tissues is low, a PDE4 inhibitor might not elevate cAMP by an amount or at a rate necessary for its therapeutic activity to be realized. One approach to obviate this possibility is to combine a PDE4 inhibitor/ICS combination therapy with a receptor agonist that drives cAMP synthesis. Clearly, from a COPD perspective, a β_2 -adrenoceptor agonist would be the obvious choice given that both LABAs and SABAs (on an as-needed basis) are a recommended treatment option for patients with mild disease (see O'Donnell et al. 2008). Adding a PDE4 inhibitor to patients with severe asthma who might not be adequately controlled by a LABA/ICS combination therapy could also afford additional benefit. Thus, a persuasive argument can be made for the development of triple combination therapies. Such novel medicines could also be advantageous in smoking asthmatic individuals in whom ICSs are relatively refractory (Chalmers et al. 2002; Thomson et al. 2004, 2006; Thomson and Spears 2005).

3.3 *Other PDE4 Inhibitor/Nuclear Hormone Receptor Combinations*

In the above sections, the potential for combining PDE4 inhibitors with a glucocorticoid or, indeed, a LABA/glucocorticoid combination was considered. However, it is clear that many NHRs, including the progesterone receptor (PR), androgen receptor (AR), and estrogen receptor may, in the presence of their cognate ligands, repress proinflammatory transcriptional responses and proinflammatory gene expression (Kalkhoven et al. 1996; McKay and Cidlowski 1999). Likewise, many of the more recently characterized NHRs including the liver X receptor, peroxisome proliferator-activated receptors (PPARs), and the various retinoic acid receptors (RARs and RXRs) are also capable of downregulating proinflammatory genes and are, consequently, being considered as alternative therapeutic options to glucocorticoids (Farrow 2008). However, in addition to identifying novel anti-inflammatory molecules, these advances also provide a trove of opportunity to develop novel NHR-based combination therapies for the treatment of inflammatory diseases (Ogawa et al. 2005). For example, like the GR, transcriptional activity of each of the PR, the mineralocorticoid receptor (MR), the RAR α , and the AR have previously been shown to be enhanced by elevated cAMP (Beck et al. 1992; Gaillard et al. 2006; Kim et al. 2005; Massaad et al. 1999; Sartorius et al. 1993). Moreover, there is evidence that agonists of PPAR γ may show enhanced ability to repress proinflammatory gene expression when combined with a LABA (Nie et al. 2005a). Consequently, the idea that PDE4 inhibitors may be paired with activators of NHRs other than GR is logical. Currently, it is unclear how widespread such effects would be and caution is merited. Indeed, LXR-mediated transcription is inhibited by activation of the cAMP/PKA cascade (Yamamoto et al. 2007b). Nevertheless, the PDE4 inhibitor, piclamilast (RP 73401), enhances transcriptional activation by RAR α and it seems very likely that the inhibition of PDE4 may provide a mechanism by which the ability of many NHR ligands can, therapeutically, be augmented (Parrella et al. 2004). Certainly, this provides impetus for analyzing potential beneficial interactions between PDE4 inhibitors and activators of NHRs.

4 **Concluding Remarks**

PDE4 inhibitors have been in clinical development for many years and it is, perhaps, gratifying that the benzamide, roflumilast, which is being developed jointly by Nycomed (formerly Altana) in Europe and Forest Research Institute in the USA, was recently approved by the European Medicines Agency Committee for Medicinal Products for Human Use (http://www.ema.europa.eu/pdfs/human/opinion/Daxas_15986110en.pdf) for the “*maintenance treatment of severe chronic obstructive pulmonary disease associated with chronic bronchitis in adult patients*”

with a history of frequent exacerbations as add-on to bronchodilator treatment” (Giembycz and Field 2010; Gross et al. 2010). However, gastrointestinal, psychiatric, and oncological adverse are significant safety issues. In this chapter, we have described several potential means by which the therapeutically beneficial actions of PDE4 inhibitors could be realized without necessarily increasing the frequency of adverse events. Thus, although a PDE4 inhibitor, roflumilast, has now reached the market for the treatment of a subpopulation of patients with severe COPD (Giembycz and Field 2010; Gross et al. 2010), the potential ability of these drugs to enhance glucocorticoid action cannot be overstated. In this respect, it seems likely that PDE4 inhibitors might, ultimately, be best exploited as part of a combination therapy with ICSs and LABAs. The possibility that PDE inhibitors might be developed for COPD (and possibly asthma) that target multiple enzyme families is not an option that has obviously been embraced by the pharmaceutical industry. Nonetheless, it is the opinion of the authors that such an approach could be rewarding. Indeed, nonselective PDE inhibitors will target many components of these multifactorial diseases at which selective PDE4 inhibitors alone do not.

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References

- Alp S, Skrygan M, Schmidt WE, Bastian A (2006) Sildenafil improves hemodynamic parameters in COPD – an investigation of six patients. *Pulm Pharmacol Ther* 19:386–390
- Ammit AJ, Hoffman RK, Amrani Y, Lazaar AL, Hay DW, Torphy TJ, Penn RB, Panettieri RA (2000) Tumor necrosis factor- α -induced secretion of RANTES and interleukin-6 from human airway smooth-muscle cells. Modulation by cyclic adenosine monophosphate. *Am J Respir Cell Mol Biol* 23:794–802
- Ammit AJ, Lazaar AL, Irani C, O’Neill GM, Gordon ND, Amrani Y, Penn RB, Panettieri RA (2002) Tumor necrosis factor- α -induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells: modulation by glucocorticoids and β -agonists. *Am J Respir Cell Mol Biol* 26:465–474

- Amsallem E, Kasparian C, Haddour G, Boissel JP, Nony P (2005) Phosphodiesterase III inhibitors for heart failure. *Cochrane Database Syst Rev* CD002230
- Amsterdam A, Sasson R (2002) The anti-inflammatory action of glucocorticoids is mediated by cell type specific regulation of apoptosis. *Mol Cell Endocrinol* 189:1–9
- Aoki M, Fukunaga M, Sugimoto T, Hirano Y, Kobayashi M, Honda K, Yamada T (2001) Studies on mechanisms of low emetogenicity of YM976, a novel phosphodiesterase type 4 inhibitor. *J Pharmacol Exp Ther* 298:1142–1149
- Aoshiba K, Nagai A (2004) Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. *Clin Rev Allergy Immunol* 27:35–44
- Banner KH, Press NJ (2009) Dual PDE3/4 inhibitors as therapeutic agents for chronic obstructive pulmonary disease. *Br J Pharmacol* 157:892–906
- Bardin PG, Dorward MA, Lampe FC, Franke B, Holgate ST (1998) Effect of selective phosphodiesterase 3 inhibition on the early and late asthmatic responses to inhaled allergen. *Br J Clin Pharmacol* 45:387–391
- Barnes PJ (2003) Theophylline: new perspectives for an old drug. *Am J Respir Crit Care Med* 167:813–818
- Barnes PJ (2006) How corticosteroids control inflammation. *Br J Pharmacol* 148:245–254
- Beck CA, Weigel NL, Edwards DP (1992) Effects of hormone and cellular modulators of protein phosphorylation on transcriptional activity, DNA binding, and phosphorylation of human progesterone receptors. *Mol Endocrinol* 6:607–620
- Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M (2003) Airway structural alterations selectively associated to severe asthma. *Am J Respir Crit Care Med* 167:1360–1368
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Bentley JK, Kadlecik A, Sherbert CH, Seger D, Sonnenburg WK, Charbonneau H, Novack JP, Beavo JA (1992) Molecular cloning of cDNA encoding a “63”-kDa calmodulin-stimulated phosphodiesterase from bovine brain. *J Biol Chem* 267:18676–18682
- Bharani A, Mathew V, Sahu A, Lunia B (2003) The efficacy and tolerability of sildenafil in patients with moderate-to-severe pulmonary hypertension. *Indian Heart J* 55:55–59
- Blease K, Burke-Gaffney A, Hellewell PG (1998) Modulation of cell adhesion molecule expression and function on human lung microvascular endothelial cells by inhibition of phosphodiesterases 3 and 4. *Br J Pharmacol* 124:229–237
- Bodor N (1984) Soft drugs: principles and methods for the design of safe drugs. *Med Res Rev* 4:449–469
- Boswell-Smith V, Spina D, Oxford AW, Comer MB, Seeds EA, Page CP (2006) The pharmacology of two novel long-acting phosphodiesterase 3/4 inhibitors, RPL554 [9, 10-dimethoxy-2(2, 4, 6-trimethyl-phenylimino)-3-(N-carbamoyl-2-aminoethyl)-3, 4, 6, 7-tetrahydro-2H-pyrimido[6, 1-a]isoquinolin-4-one] and RPL565 [6, 7-dihydro-2-(2, 6-diisopropylphenoxy)-9, 10-dimethoxy-4H-pyrimido[6, 1-a]isoquinolin-4-one]. *J Pharmacol Exp Ther* 318:840–848
- Brunnee T, Engelstatter R, Steijnmans VW, Kunkel G (1992) Bronchodilatory effect of inhaled zardaverine, a phosphodiesterase III and IV inhibitor, in patients with asthma. *Eur Respir J* 5:982–985
- Bruss MD, Richter W, Horner K, Jin SL, Conti M (2008) Critical role of PDE4D in β_2 -adrenoceptor-dependent cAMP signaling in mouse embryonic fibroblasts. *J Biol Chem* 283:22430–22442
- Bureau Y, Handa M, Zhu Y, Laliberte F, Moore CS, Liu S, Huang Z, MacDonald D, Xu DG, Robertson GS (2006) Neuroanatomical and pharmacological assessment of *Fos* expression induced in the rat brain by the phosphodiesterase-4 inhibitor, 6-(4-pyridylmethyl)-8-(3-nitrophenyl) quinoline. *Neuropharmacology* 51:974–985
- Burgin AB, Magnusson OT, Singh J, Witte P, Staker BL, Bjornsson JM, Thorsteinsdottir M, Hrafnisdottir S, Hagen T, Kiselyov AS, Stewart LJ, Gurney ME (2010) Design of phosphodiesterase 4D (PDE4D) allosteric modulators for enhancing cognition with improved safety. *Nat Biotechnol* 28:63–70
- Burnouf C, Pruniaux MP (2002) Recent advances in PDE4 inhibitors as immunoregulators and anti-inflammatory drugs. *Curr Pharm Des* 8:1255–1296

- Burnouf C, Pruniaux MP, Szilagy CM (1998) Phosphodiesterase 4 inhibitors. *Ann Rev Med Chem* 33:91–109
- Calverley PMA, Sanchez-Toril F, McIvor A, Teichman P, Bredenbrocker D, Fabbri LM (2007) Effect of one year treatment with roflumilast in severe chronic obstructive lung disease. *Am J Respir Crit Care Med* 176:151–161
- Calverley PMA, Rabe KF, Goehring U-M, Kristiansen S, Fabbri LM, Martinez FJ (2009) Roflumilast in symptomatic chronic obstructive pulmonary disease: two randomized clinical trials. *Lancet* 374:684–695
- Celli BR, Thomas NE, Anderson JA, Ferguson GT, Jenkins CR, Jones PW, Vestbo J, Knobil K, Yates JC, Calverley PM (2008) Effect of pharmacotherapy on rate of decline of lung function in chronic obstructive pulmonary disease: results from the TORCH study. *Am J Respir Crit Care Med* 178:332–338
- Chalmers GW, Macleod KJ, Little SA, Thomson LJ, McSharry CP, Thomson NC (2002) Influence of cigarette smoking on inhaled corticosteroid treatment in mild asthma. *Thorax* 57: 226–230
- Chaouat A, Naeije R, Weitzenblum E (2008) Pulmonary hypertension in COPD. *Eur Respir J* 32: 1371–1385
- Charan NB (2001) Does sildenafil also improve breathing? *Chest* 120:305–306
- Chockalingam A, Gnanavelu G, Venkatesan S, Elangovan S, Jagannathan V, Subramaniam T, Alagesan R, Dorairajan S (2005) Efficacy and optimal dose of sildenafil in primary pulmonary hypertension. *Int J Cardiol* 99:91–95
- Clark AR (2007) Anti-inflammatory functions of glucocorticoid-induced genes. *Mol Cell Endocrinol* 275:79–97
- Clarke DL, Belvisi MG, Smith SJ, Hardaker E, Yacoub MH, Meja KK, Rewton R, Slater DM, Giembycz MA (2005a) Prostanoid receptor expression by human airways smooth muscle cells and regulation of the secretion of granulocyte colony-stimulating factor. *Am J Physiol*. 288: L238–L250
- Clarke DL, Belvisi MG, Hardaker E, Newton R, Giembycz MA (2005b) E-Ring 8-isoprostanes are agonists at EP₂- and EP₄- prostanoid receptors on human airways smooth muscle cells and regulate the release of colony-stimulating factors by activating cAMP-dependent protein kinase. *Mol Pharmacol*. 67:383–393
- Cordova C, Musca A, Violi F, Alessandri C, Perrone A, Balsano F (1985) Platelet hyperfunction in patients with chronic airways obstruction. *Eur J Respir Dis* 66:9–12
- Dahlman-Wright K, Wright A, Gustafsson JA, Carlstedt-Duke J (1991) Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. *J Biol Chem* 266:3107–3112
- De Bosscher K, Vanden Berghe W, Haegeman G (2003) The interplay between the glucocorticoid receptor and nuclear factor- κ B or activator protein-1: molecular mechanisms for gene repression. *Endocr Rev* 24:488–522
- Diefenbacher M, Sekula S, Heilbock C, Maier JV, Litfin M, van Dam H, Castellazzi M, Herrlich P, Kassel O (2008) Restriction to *Fos* family members of Trip6-dependent coactivation and glucocorticoid receptor-dependent transrepression of activator protein-1. *Mol Endocrinol* 22: 1767–1780
- Doherty AM (1999) Phosphodiesterase 4 inhibitors as novel anti-inflammatory agents. *Curr Opin Chem Biol* 3:466–473
- Dong H, Zitt C, Auriga C, Hatzelmann A, Epstein PM (2010) Inhibition of PDE3, PDE4 and PDE7 potentiates glucocorticoid-induced apoptosis and overcomes glucocorticoid resistance in CEM T leukemic cells. *Biochem Pharmacol* 79:321–329
- Dunnill MS, Massarella GR, Anderson JA (1969) A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* 24:176–179
- Duplantier AJ, Biggers MS, Chambers RJ, Cheng JB, Cooper K, Damon DB, Egger JF, Kraus KG, Marfat A, Masamune H, Pillar JS, Shirley JT, UmLand JP, Watson JW (1996)

- Biarylcarboxylic acids and -amides: inhibition of phosphodiesterase type IV versus [³H]rolipram binding activity and their relationship to emetic behavior in the ferret. *J Med Chem* 39: 120–125
- Dyke HJ, Montana JG (2002) Update on the therapeutic potential of PDE4 inhibitors. *Expert Opin Investig Drugs* 11:1–13
- Ebina M, Takahashi T, Chiba T, Motomiya M (1993) Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3D morphometric study. *Am Rev Respir Dis* 148:720–726
- Eddleston J, Herschbach J, Wagelie-Steffen AL, Christiansen SC, Zuraw BL (2007) The anti-inflammatory effect of glucocorticoids is mediated by glucocorticoid-induced leucine zipper in epithelial cells. *J Allergy Clin Immunol* 119:115–122
- Edmondson SD, Mastracchio A, He J, Chung CC, Forrest MJ, Hofsess S, MacIntyre E, Metzger J, O'Connor N, Patel K, Tong X, Tota MR, Van der Ploeg LH, Varnerin JP, Fisher MH, Wyvratt MJ, Weber AE, Parmee ER (2003) Benzyl vinylogous amide substituted aryldihydropyridazinones and aryldimethylpyrazolones as potent and selective PDE3B inhibitors. *Bioorg Med Chem Lett* 13:3983–3987
- Epstein PM, Moraski S Jr, Hachisu R (1987) Identification and characterization of a Ca²⁺-calmodulin-sensitive cyclic nucleotide phosphodiesterase in a human lymphoblastoid cell line. *Biochem J* 243:533–539
- Evans DJ, Taylor DA, Zetterstrom O, Chung KF, O'Connor BJ, Barnes PJ (1997) A comparison of low-dose inhaled budesonide plus theophylline and high-dose inhaled budesonide for moderate asthma. *N Engl J Med* 337:1412–1418
- Ezeamuzie CI (2001) Requirement of additional adenylate cyclase activation for the inhibition of human eosinophil degranulation by phosphodiesterase IV inhibitors. *Eur J Pharmacol* 417: 11–18
- Farrow SN (2008) Nuclear receptors: doubling up in the lung. *Curr Opin Pharmacol* 8:275–279
- Fischer TA, Palmethofer A, Gambaryan S, Butt E, Jassoy C, Walter U, Sopper S, Lohmann SM (2001) Activation of cGMP-dependent protein kinase I β inhibits interleukin 2 release and proliferation of T cell receptor-stimulated human peripheral T cells. *J Biol Chem* 276: 5967–5974
- Fujimura M, Kamio Y, Saito M, Hashimoto T, Matsuda T (1995) Bronchodilator and bronchoprotective effects of cilostazol in humans in vivo. *Am J Respir Crit Care Med* 151: 222–225
- Gaillard E, Bruck N, Brelivet Y, Bour G, Lalevee S, Bauer A, Poch O, Moras D, Rochette-Egly C (2006) Phosphorylation by PKA potentiates retinoic acid receptor α activity by means of increasing interaction with and phosphorylation by cyclin H/cdk7. *Proc Natl Acad Sci USA* 103:9548–9553
- Galie N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, Fleming T, Parpia T, Burgess G, Branzi A, Grimminger F, Kurzyna M, Simonneau G (2005) Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med* 353:2148–2157
- Gamble E, Grootendorst DC, Brightling CE, Troy S, Qiu Y, Zhu J, Parker D, Matin D, Majumdar S, Vignola AM, Kroegel C, Morell F, Hansel TT, Rennard SI, Compton C, Amit O, Tat T, Edelson J, Pavord ID, Rabe KF, Barnes NC, Jeffery PK (2003) Anti-inflammatory effects of the phosphodiesterase-4 inhibitor cilomilast (*Ariflo*) in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 168:976–982
- Gantner F, Gotz C, Gekeler V, Schudt C, Wendel A, Hatzelmann A (1998) Phosphodiesterase profile of human B lymphocytes from normal and atopic donors and the effects of PDE inhibition on B cell proliferation. *Br J Pharmacol* 123:1031–1038
- Gantner F, Schudt C, Wendel A, Hatzelmann A (1999) Characterization of the phosphodiesterase (PDE) pattern of in vitro - generated human dendritic cells (DC) and the influence of PDE inhibitors on DC function. *Pulm Pharmacol Ther* 12:377–386
- Ghofrani HA, Voswinckel R, Reichenberger F, Olschewski H, Haredza P, Karadas B, Schermuly RT, Weissmann N, Seeger W, Grimminger F (2004) Differences in hemodynamic

and oxygenation responses to three different phosphodiesterase-5 inhibitors in patients with pulmonary arterial hypertension: a randomized prospective study. *J Am Coll Cardiol* 44: 1488–1496

Gibson LC, Hastings SF, McPhee I, Clayton RA, Darroch CE, Mackenzie A, Mackenzie FL, Nagasawa M, Stevens PA, MacKenzie SJ (2006) The inhibitory profile of ibudilast against the human phosphodiesterase enzyme family. *Eur J Pharmacol* 538:39–42

Giembycz MA (2000) Phosphodiesterase 4 inhibitors and the treatment of asthma: where are we now and where do we go from here? *Drugs* 59:193–212

Giembycz MA (2001) Cilomilast: a second generation phosphodiesterase 4 inhibitor for asthma and chronic obstructive pulmonary disease. *Expert Opin Investig Drugs* 10:1361–1379

Giembycz MA (2005a) Life after PDE4: overcoming adverse events with dual-specificity phosphodiesterase inhibitors. *Curr Opin Pharmacol* 5:238–244

Giembycz MA (2005b) Phosphodiesterase 4: Selective and dual-specificity inhibitors for the therapy of chronic obstructive pulmonary disease. *Proc Am Thor Soc* 2:326–333

Giembycz MA (2006) An update and appraisal of the cilomilast phase III clinical development programme for chronic obstructive pulmonary disease. *Br J Clin Pharmacol* 62:138–152

Giembycz MA (2008) Can the anti-inflammatory potential of PDE4 inhibitors be realized: guarded optimism or wishful thinking? *Br J Pharmacol* 155:288–290

Giembycz MA, Barnes PJ (1991) Selective inhibition of a high affinity type IV cyclic AMP phosphodiesterase in bovine trachealis by AH 21-132. Relevance to the spasmolytic and anti-spasmodic actions of AH 21-132 in the intact tissue. *Biochem Pharmacol* 42:663–677

Giembycz MA, Field SK (2010) Roflumilast: first in class phosphodiesterase 4 inhibitor for treatment of COPD. *Drug Des Dev Ther* 4:147–158

Giembycz MA, Newton R (2006) Beyond the dogma: novel β_2 -adrenoceptor signalling in the airways. *Eur Respir J* 27:1286–1306

Giembycz MA, Smith SJ (2006a) Phosphodiesterase 7 as a therapeutic target. *Drugs Future* 31: 207–230

Giembycz MA, Smith SJ (2006b) Phosphodiesterase 7A: a new therapeutic target for alleviating chronic inflammation? *Curr Pharm Des* 12:3207–3220

Giembycz MA, Corrigan CJ, Seybold J, Newton R, Barnes PJ (1996) Identification of cyclic AMP phosphodiesterases 3, 4 and 7 in human CD4⁺ and CD8⁺ T-lymphocytes: role in regulating proliferation and the biosynthesis of interleukin-2. *Br J Pharmacol* 118:1945–1958

Giembycz MA, Kaur M, Leigh R, Newton R (2008) A Holy Grail of asthma management: toward understanding how long-acting β_2 -adrenoceptor agonists enhance the clinical efficacy of inhaled corticosteroids. *Br J Pharmacol* 153:1090–1104

Gil C, Campillo NE, Perez DI, Martinez A (2008) PDE7 inhibitors as new drugs for neurological and inflammatory disorders. *Exp Opin Ther Patents* 18:1127–1139

Goto M, Murakawa M, Kadoshima-Yamaoka K, Tanaka Y, Inoue H, Murafuji H, Hayashi Y, Miura K, Nakatsuka T, Nagahira K, Chamoto K, Fukuda Y, Nishimura T (2009) Phosphodiesterase 7A inhibitor ASB16165 suppresses proliferation and cytokine production of NKT cells. *Cell Immunol* 258:147–151

Grootendorst DC, Gauw SA, Baan R, Kelly J, Murdoch RD, Sterk PJ, Rabe KF (2003) Does a single dose of the phosphodiesterase 4 inhibitor, cilomilast (15 mg), induce bronchodilation in patients with chronic obstructive pulmonary disease? *Pulm Pharmacol Ther* 16: 115–120

Grootendorst DC, Gauw SA, Verhoosel RM, Sterk PJ, Hospers JJ, Bredenbroeker D, Bethke TD, Hiemstra PS, Rabe KF (2007) The PDE4 inhibitor roflumilast reduces sputum neutrophil and eosinophil numbers in patients with COPD. *Thorax* 62:1081–1087

Gross NJ, Giembycz MA, Rennard SI (2010) Treatment of chronic obstructive pulmonary disease with roflumilast - a new phosphodiesterase 4 inhibitor. *J COPD* 7:141–153

Growcott EJ, Spink KG, Ren X, Afzal S, Banner KH, Wharton J (2006) Phosphodiesterase type 4 expression and anti-proliferative effects in human pulmonary artery smooth muscle cells. *Respir Res* 7:9

- Hallsworth MP, Twort CH, Lee TH, Hirst SJ (2001) β_2 -adrenoceptor agonists inhibit release of eosinophil-activating cytokines from human airway smooth muscle cells. *Br J Pharmacol* 132: 729–741
- Heard BE, Hossain S (1973) Hyperplasia of bronchial muscle in asthma. *J Pathol* 110:319–331
- Hertz AL, Bender AT, Smith KC, Gilchrist M, Amieux PS, Aderem A, Beavo JA (2009) Elevated cyclic AMP and PDE4 inhibition induce chemokine expression in human monocyte-derived macrophages. *Proc Natl Acad Sci USA* 106:21978–21983
- Hirst SJ, Martin JG, Bonacci JV, Chan V, Fixman ED, Hamid QA, Herszberg B, Lavoie JP, McVicker CG, Moir LM, Nguyen TT, Peng Q, Ramos-Barbon D, Stewart AG (2004) Proliferative aspects of airway smooth muscle. *J Allergy Clin Immunol* 114:S2–S17
- Hogg JC (1996) Airway behavior and its regulation: how do structural changes affect airway behavior? *Am J Respir Crit Care Med* 153:S16–S18
- Hogg JC (2004) Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet* 364:709–721
- Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Pare PD (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 350:2645–2653
- Holden NS, Rider CF, Bell MJ, Velayudhan J, King EM, Kaur M, Salmon M, Giembycz MA, Newton R (2010) Enhancement of inflammatory mediator release by β_2 -adrenoceptor agonists in airway epithelial cells is reversed by glucocorticoid action. *Br J Pharmacol* 160: 410–420
- Holverda S, Rietema H, Bogaard HJ, Westerhof N, Postmus PE, Boonstra A, Vonk-Noordegraaf A (2008) Acute effects of sildenafil on exercise pulmonary hemodynamics and capacity in patients with COPD. *Pulm Pharmacol Ther* 21:558–564
- Hossain S, Heard BE (1970) Hyperplasia of bronchial muscle in chronic bronchitis. *J Pathol* 101: 171–184
- Houslay MD, Schafer P, Zhang KY (2005) Phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10:1503–1519
- Howarth PH, Beckett P, Dahl R (2000) The effect of long-acting β_2 -agonists on airway inflammation in asthmatic patients. *Respir Med* 94(Suppl F):S22–S25
- Huang Z, Ducharme Y, MacDonald D, Robichaud A (2001) The next generation of PDE4 inhibitors. *Curr Opin Chem Biol* 5:432–438
- Hurwitz RL, Hirsch KM, Clark DJ, Holcombe VN, Hurwitz MY (1990) Induction of a calcium/calmodulin-dependent phosphodiesterase during phytohemagglutinin-stimulated lymphocyte mitogenesis. *J Biol Chem* 265:8901–8907
- Ito K, Barnes PJ, Adcock IM (2000) Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1 β -induced histone H4 acetylation on lysines 8 and 12. *Mol Cell Biol* 20: 6891–6903
- Jeffery PK (2001) Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 164:S28–S38
- Jenkins CR, Jones PW, Calverley PM, Celli B, Anderson JA, Ferguson GT, Yates JC, Willits LR, Vestbo J (2009) Efficacy of salmeterol/fluticasone propionate by GOLD stage of chronic obstructive pulmonary disease: analysis from the randomised, placebo-controlled TORCH study. *Respir Res* 10:59
- Jiang X, Li J, Paskind M, Epstein PM (1996) Inhibition of calmodulin-dependent phosphodiesterase induces apoptosis in human leukemic cells. *Proc Natl Acad Sci USA* 93:11236–11241
- Jones NA, Leport M, Holand T, Vos T, Morgan M, Fink M, Pruniaux MP, Berthelie C, O'Connor BJ, Bertrand C, Page CP (2006) Phosphodiesterase (PDE) 7 in inflammatory cells from patients with asthma and COPD. *Pulm Pharmacol Ther* 20:60–68
- Joseph EC (2000) Arterial lesions induced by phosphodiesterase III (PDE III) inhibitors and D₁ agonists. *Toxicol Lett* 112–113:537–546
- Jyothula S, Safdar Z (2009) Update on pulmonary hypertension complicating chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis* 4:351–363

- Kadoshima-Yamaoka K, Goto M, Murakawa M, Yoshioka R, Tanaka Y, Inoue H, Murafuji H, Kanki S, Hayashi Y, Nagahira K, Ogata A, Nakatsuka T, Fukuda Y (2009a) ASB16165, a phosphodiesterase 7A inhibitor, reduces cutaneous TNF α level and ameliorates skin edema in phorbol ester 12-O-tetradecanoylphorbol-13-acetate-induced skin inflammation model in mice. *Eur J Pharmacol* 613:163–166
- Kadoshima-Yamaoka K, Murakawa M, Goto M, Tanaka Y, Inoue H, Murafuji H, Hayashi Y, Nagahira K, Miura K, Nakatsuka T, Chamoto K, Fukuda Y, Nishimura T (2009b) Effect of phosphodiesterase 7 inhibitor ASB16165 on development and function of cytotoxic T lymphocyte. *Int Immunopharmacol* 9:97–102
- Kadoshima-Yamaoka K, Murakawa M, Goto M, Tanaka Y, Inoue H, Murafuji H, Nagahira A, Hayashi Y, Nagahira K, Miura K, Nakatsuka T, Chamoto K, Fukuda Y, Nishimura T (2009c) ASB16165, a novel inhibitor for phosphodiesterase 7A (PDE7A), suppresses IL-12-induced IFN-gamma production by mouse activated T lymphocytes. *Immunol Lett* 122:193–197
- Kalkhoven E, Wissink S, van der Saag PT, van der Burg B (1996) Negative interaction between the RelA(p65) subunit of NF- κ B and the progesterone receptor. *J Biol Chem* 271:6217–6224
- Kaur M, Chivers JE, Giembycz MA, Newton R (2008a) Long-acting β_2 -adrenoceptor agonists synergistically enhance glucocorticoid-dependent transcription in human airway epithelial and smooth muscle cells. *Mol Pharmacol* 73:201–214
- Kaur M, Holden NS, Wilson SM, Sukkar MB, Chung KF, Barnes PJ, Newton R, Giembycz MA (2008b) Effect of β_2 -adrenoceptor agonists and other cAMP-elevating agents on inflammatory gene expression in human ASM cells: a role for protein kinase A. *Am J Physiol Lung Cell Mol Physiol* 295:L505–L514
- Kim J, Jia L, Stallcup MR, Coetzee GA (2005) The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus. *J Mol Endocrinol* 34:107–118
- King EM, Holden NS, Gong W, Rider CF, Newton R (2009) Inhibition of NF- κ B-dependent transcription by MKP-1: transcriptional repression by glucocorticoids occurring via p38 MAPK. *J Biol Chem* 284:26803–26815
- Kita T, Fujimura M, Myou S, Watanabe K, Waseda Y, Nakao S (2009) Effects of KF19514, a phosphodiesterase 4 and 1 inhibitor, on bronchial inflammation and remodeling in a murine model of chronic asthma. *Allergol Int* 58:267–275
- Kvien TK, Fjeld E, Slatkowsky-Christensen B, Nichols M, Zhang Y, Proven A, Mikkelsen K, Palm O, Borisy AA, Lessem J (2008) Efficacy and safety of a novel synergistic drug candidate, CRx-102, in hand osteoarthritis. *Ann Rheum Dis* 67:942–948
- Lazaar AL, Panettieri RA (2003) Is airway remodeling clinically relevant in asthma? *Am J Med* 115:652–659
- Leeman M, Lejeune P, Melot C, Naeije R (1987) Reduction in pulmonary hypertension and in airway resistances by enoximone (MDL 17, 043) in decompensated COPD. *Chest* 91:662–666
- Lepore JJ, Maroo A, Bigatello LM, Dec GW, Zapol WM, Bloch KD, Semigran MJ (2005) Hemodynamic effects of sildenafil in patients with congestive heart failure and pulmonary hypertension: combined administration with inhaled nitric oxide. *Chest* 127:1647–1653
- Lim S, Jatakanon A, Gordon D, MacDonald C, Chung KF, Barnes PJ (2000) Comparison of high dose inhaled steroids, low dose inhaled steroids plus low dose theophylline, and low dose inhaled steroids alone in chronic asthma in general practice. *Thorax* 55:837–841
- MacDonald D, Perrier H, Liu S, Laliberte F, Rasori R, Robichaud A, Masson P, Huang Z (2000) Hunting the emesis and efficacy targets of PDE4 inhibitors: identification of the photoaffinity probe 8-(3-azidophenyl)-6-[(4-iodo-1H-1-imidazolyl)methyl]quinoline (APIIMQ). *J Med Chem* 43:3820–3823
- Madden BP, Allenby M, Loke TK, Sheth A (2006) A potential role for sildenafil in the management of pulmonary hypertension in patients with parenchymal lung disease. *Vascul Pharmacol* 44:372–376

- Manabe H, Akuta K, Sejimo H, Kawasaki H, Nukui E, Ichimura M, Kase H, Kawakita T, Suzuki F, Kitamura S, Sato S, Ohmori K (1997) Anti-inflammatory and bronchodilator properties of KFI9514, a phosphodiesterase 4 and 1 inhibitor. *Eur J Pharmacol* 332:97–107
- Martin JG, Duguet A, Eidelman DH (2000) The contribution of airway smooth muscle to airway narrowing and airway hyperresponsiveness in disease. *Eur Respir J* 16:349–354
- Massaad C, Houard N, Lombes M, Barouki R (1999) Modulation of human mineralocorticoid receptor function by protein kinase A. *Mol Endocrinol* 13:57–65
- McKay LI, Cidlowski JA (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor- κ B and steroid receptor-signaling pathways. *Endocr Rev* 20: 435–459
- Meja K, Catley MC, Cambridge LM, Barnes PJ, Lum H, Newton R, Giembycz MA (2004) Adenovirus-mediated delivery and expression of a cAMP-dependent protein kinase inhibitor gene to BEAS-2B epithelial cells abolishes the anti-inflammatory effects of rolipram, salbutamol and prostaglandin E₂: a comparison with H-89. *J Pharmacol Exp Ther* 309:833–844
- Meliton AY, Munoz NM, Liu J, Lambertino AT, Boetticher E, Myo S, Myou S, Zhu X, Johnson M, Leff AR (2003) Blockade of LTC₄ synthesis caused by additive inhibition of gIV-PLA₂ phosphorylation: Effect of salmeterol and PDE4 inhibition in human eosinophils. *J Allergy Clin Immunol* 112:404–410
- Meliton AY, Munoz NM, Lambertino A, Boetticher E, Learoyd J, Zhu X, Leff AR (2006) Phosphodiesterase 4 inhibition of β 2-integrin adhesion caused by leukotriene B₄ and TNF α in human neutrophils. *Eur Respir J* 28:920–928
- Michaeli T, Bloom TJ, Martins T, Loughney K, Ferguson K, Riggs M, Rodgers L, Beavo JA, Wigler M (1993) Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase-deficient *Saccharomyces cerevisiae*. *J Biol Chem* 268:12925–12932
- Michelakis E, Tymchak W, Lien D, Webster L, Hashimoto K, Archer S (2002) Oral sildenafil is an effective and specific pulmonary vasodilator in patients with pulmonary arterial hypertension: comparison with inhaled nitric oxide. *Circulation* 105:2398–2403
- Michelakis ED, Tymchak W, Noga M, Webster L, Wu XC, Lien D, Wang SH, Modry D, Archer SL (2003) Long-term treatment with oral sildenafil is safe and improves functional capacity and hemodynamics in patients with pulmonary arterial hypertension. *Circulation* 108: 2066–2069
- Miller AH, Vogt GJ, Pearce BD (2002) The phosphodiesterase type 4 inhibitor, rolipram, enhances glucocorticoid receptor function. *Neuropsychopharmacology* 27:939–948
- Mittelstadt PR, Ashwell JD (2001) Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. *J Biol Chem* 276:29603–29610
- Movsesian MA (2003) PDE3 inhibition in dilated cardiomyopathy: reasons to reconsider. *J Card Fail* 9:475–480
- Myou S, Fujimura M, Kamio Y, Ishiura Y, Tachibana H, Hirose T, Hashimoto T, Matsuda T (1999) Bronchodilator effect of inhaled olprinone, a phosphodiesterase 3 inhibitor, in asthmatic patients. *Am J Respir Crit Care Med* 160:817–820
- Naeije R (2003) Pulmonary hypertension and right heart failure in COPD. *Monaldi Arch Chest Dis* 59:250–253
- Naeije R, Barbera JA (2001) Pulmonary hypertension associated with COPD. *Crit Care* 5: 286–289
- Nakata A, Ogawa K, Sasaki T, Koyama N, Wada K, Kotera J, Kikkawa H, Omori K, Kaminuma O (2002) Potential role of phosphodiesterase 7 in human T cell function: comparative effects of two phosphodiesterase inhibitors. *Clin Exp Immunol* 128:460–466
- Newton R (2000) Molecular mechanisms of glucocorticoid action: what is important? *Thorax* 55: 603–613
- Newton R, Holden NS (2007) Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? *Mol Pharmacol* 72:799–809

- Newton R, Leigh R, Giembycz MA (2010) Pharmacological strategies for improving the efficacy and therapeutic ratio of glucocorticoids in inflammatory lung diseases. *Pharmacol Ther* 125: 286–327
- Nie M, Corbett L, Knox AJ, Pang L (2005a) Differential regulation of chemokine expression by peroxisome proliferator-activated receptor γ agonists: interactions with glucocorticoids and β_2 -agonists. *J Biol Chem* 280:2550–2561
- Nie M, Knox AJ, Pang L (2005b) β_2 -Adrenoceptor agonists, like glucocorticoids, repress eotaxin gene transcription by selective inhibition of histone H4 acetylation. *J Immunol* 175:478–486
- O'Donnell DE, Hernandez P, Kaplan A, Aaron S, Bourbeau J, Marciniuk D, Balter M, Ford G, Gervais A, Lacasse Y, Maltais F, Road J, Rocker G, Sin D, Sinuff T, Voduc N (2008) Canadian Thoracic Society recommendations for management of chronic obstructive pulmonary disease - 2008 update - highlights for primary care. *Can Respir J* 15(Suppl A):1A–8A
- Ogawa R, Streiff MB, Bugayenko A, Kato GJ (2002) Inhibition of PDE4 phosphodiesterase activity induces growth suppression, apoptosis, glucocorticoid sensitivity, p53, and p21^(WAF1/CIP1) proteins in human acute lymphoblastic leukemia cells. *Blood* 99:3390–3397
- Ogawa S, Lozach J, Benner C, Pascual G, Tangirala RK, Westin S, Hoffmann A, Subramaniam S, David M, Rosenfeld MG, Glass CK (2005) Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell* 122:707–721
- Oxford AW, Jack D (2000) Derivatives of pyrimidino[6,1-*a*]isoquinoline-4-one. Verona Pharma, WO 00/58308
- Pages L, Gavalda A, Lehner MD (2009) PDE4 inhibitors: a review of current developments (2005–2009). *Expert Opin Ther Pat* 19:1501–1519
- Pang L, Knox AJ (2000) Synergistic inhibition by β_2 -agonists and corticosteroids on tumor necrosis factor- α -induced interleukin-8 release from cultured human airway smooth-muscle cells. *Am J Respir Cell Mol Biol* 23:79–85
- Parrella E, Gianni' M, Ceconi V, Nigro E, Barzago MM, Rambaldi A, Rochette-Egly C, Terao M, Garattini E (2004) Phosphodiesterase IV inhibition by piclamilast potentiates the cytodifferentiating action of retinoids in myeloid leukemia cells. Cross-talk between the cAMP and the retinoic acid signaling pathways. *J Biol Chem* 279:42026–42040
- Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, Ullman A (1997) Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. *N Engl J Med* 337: 1405–1411
- Postma DS, Kerstjens HA (1998) Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 158:S187–S192
- Prasad S, Wilkinson J, Gatzoulis MA (2000) Sildenafil in primary pulmonary hypertension. *N Engl J Med* 343:1342
- Pratt WB, Galigniana MD, Harrell JM, DeFranco DB (2004) Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement. *Cell Signal* 16:857–872
- Rangarajan PN, Umesono K, Evans RM (1992) Modulation of glucocorticoid receptor function by protein kinase A. *Mol Endocrinol* 6:1451–1457
- Reisman D, Thompson EA (1995) Glucocorticoid regulation of cyclin D3 gene transcription and mRNA stability in lymphoid cells. *Mol Endocrinol* 9:1500–1509
- Roberts JA, Bradding P, Britten KM, Walls AF, Wilson S, Gratziau C, Holgate ST, Howarth PH (1999) The long-acting β_2 -agonist salmeterol xinafoate: effects on airway inflammation in asthma. *Eur Respir J* 14:275–282
- Robicsek SA, Blanchard DK, Djeu JY, Krzanowski JJ, Szentivanyi A, Polson JB (1991) Multiple high-affinity cAMP-phosphodiesterases in human T-lymphocytes. *Biochem Pharmacol* 42: 869–877
- Rolan P, Hutchinson M, Johnson K (2009) Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. *Expert Opin Pharmacother* 10:2897–2904
- Rybalkin SD, Bornfeldt KE, Sonnenburg WK, Rybalkina IG, Kwak KS, Hanson K, Krebs EG, Beavo JA (1997) Calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDE1C) is

- induced in human arterial smooth muscle cells of the synthetic, proliferative phenotype. *J Clin Invest* 100:2611–2621
- Rybalkin SD, Rybalkina I, Beavo JA, Bornfeldt KE (2002) Cyclic nucleotide phosphodiesterase 1C promotes human arterial smooth muscle cell proliferation. *Circ Res* 90:151–157
- Sartorius CA, Tung L, Takimoto GS, Horwitz KB (1993) Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP. *J Biol Chem* 268:9262–9266
- Sastry BK, Narasimhan C, Reddy NK, Anand B, Prakash GS, Raju PR, Kumar DN (2002) A study of clinical efficacy of sildenafil in patients with primary pulmonary hypertension. *Indian Heart J* 54:410–414
- Sastry BK, Narasimhan C, Reddy NK, Raju BS (2004) Clinical efficacy of sildenafil in primary pulmonary hypertension: a randomized, placebo-controlled, double-blind, crossover study. *J Am Coll Cardiol* 43:1149–1153
- Schacke H, Docke WD, Asadullah K (2002) Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* 96:23–43
- Schermuly RT, Pullamsetti SS, Kwapiszewska G, Dumitrescu R, Tian X, Weissmann N, Ghofrani HA, Kaulen C, Dunkern T, Schudt C, Voswinkel R, Zhou J, Samidurai A, Klepetko W, Paddenberg R, Kummer W, Seeger W, Grimminger F (2007) Phosphodiesterase I upregulation in pulmonary arterial hypertension: target for reverse-remodeling therapy. *Circulation* 115:2331–2339
- Schudt C, Tenor H, Hatzelmann A (1995) PDE isoenzymes as targets for anti-asthma drugs. *Eur Respir J* 8:1179–1183
- Secchiero P, Zella D, Curreli S, Mirandola P, Capitani S, Gallo RC, Zauli G (2000) Pivotal role of cyclic nucleoside phosphodiesterase 4 in Tat-mediated CD4⁺ T cell hyperactivation and HIV type 1 replication. *Proc Natl Acad Sci USA* 97:14620–14625
- Seldon PM, Barnes PJ, Meja K, Giembycz MA (1995) Suppression of lipopolysaccharide-induced tumor necrosis factor- α generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: interaction with stimulants of adenylyl cyclase. *Mol Pharmacol* 48:747–757
- Selige J, Tenor H, Hatzelmann A, Dunkern T (2010) Cytokine-dependent balance of mitogenic effects in primary human lung fibroblasts related to cyclic AMP signaling and phosphodiesterase 4 inhibition. *J Cell Physiol* 223:317–326
- Shrewsbury S, Pyke S, Britton M (2000) Meta-analysis of increased dose of inhaled steroid or addition of salmeterol in symptomatic asthma (MIASMA). *BMJ* 320:1368–1373
- Sindi A, Todd DC, Nair P (2009) Antiinflammatory effects of long-acting β_2 -agonists in patients with asthma: a systematic review and meta-analysis. *Chest* 136:145–154
- Singh D, Petavy F, Macdonald AJ, Lazaar AL, OC BJ (2010) The inhaled phosphodiesterase 4 inhibitor GSK256066 reduces allergen challenge responses in asthma. *Respir Res* 11:26
- Smith SJ, Brookes-Fazakerley S, Donnelly LE, Barnes PJ, Barnette MS, Giembycz MA (2003) Ubiquitous expression of phosphodiesterase 7A in human proinflammatory and immune cells. *Am J Physiol Lung Cell Mol Physiol* 284:L279–L289
- Smith SJ, Cieslinski LB, Newton R, Donnelly LE, Fenwick PS, Nicholson AG, Barnes PJ, Barnette MS, Giembycz MA (2004) Discovery of BRL 50481 [3-(N, N-dimethylsulfonamido)-4-methylnitrobenzene], a selective inhibitor of phosphodiesterase 7: In vitro studies in human monocytes, lung macrophage and CD8⁺ T-lymphocytes. *Mol Pharmacol* 66:1679–1689
- Snyder PB, Beaton G, Rueter JK, Fanning DL, Warren SD, Hadidarua SS (2002) Selective PDE3B inhibitors and uses of the same in therapy. ICOS Corporation, WO02/070469 A2
- Souness JE, Villamil ME, Scott LC, Tomkinson A, Giembycz MA, Raeburn D (1994) Possible role of cyclic AMP phosphodiesterases in the actions of ibudilast on eosinophil thromboxane generation and airways smooth muscle tone. *Br J Pharmacol* 111:1081–1088
- Souness JE, Aldous D, Sargent C (2000) Immunosuppressive and anti-inflammatory effects of cyclic AMP phosphodiesterase (PDE) type 4 inhibitors. *Immunopharmacology* 47:127–162

- Spence S, Rena G, Sweeney G, Houslay MD (1995) Induction of Ca^{2+} /calmodulin-stimulated cyclic AMP phosphodiesterase (PDE1) activity in Chinese hamster ovary cells (CHO) by phorbol 12-myristate 13-acetate and by the selective overexpression of protein kinase C isoforms. *Biochem J* 310:975–982
- Spence S, Rena G, Sullivan M, Erdogan S, Houslay MD (1997) Receptor-mediated stimulation of lipid signalling pathways in CHO cells elicits the rapid transient induction of the PDE1B isoform of Ca^{2+} /calmodulin-stimulated cAMP phosphodiesterase. *Biochem J* 321:157–163
- Srivani P, Usharani D, Jemmis ED, Sastry GN (2008) Subtype selectivity in phosphodiesterase 4 (PDE4): a bottleneck in rational drug design. *Curr Pharm Des* 14:3854–3872
- Steiner MK, Preston IR, Klinger JR, Hill NS (2005) Pulmonary hypertension: inhaled nitric oxide, sildenafil and natriuretic peptides. *Curr Opin Pharmacol* 5:245–250
- Stellato C (2004) Post-transcriptional and nongenomic effects of glucocorticoids. *Proc Am Thorac Soc* 1:255–263
- Stewart AG (2001) Airway wall remodelling and hyperresponsiveness: modelling remodelling in vitro and in vivo. *Pulm Pharmacol Ther* 14:255–265
- Sun B, Li H, Shakur Y, Hensley J, Hockman S, Kambayashi J, Manganiello VC, Liu Y (2007) Role of phosphodiesterase type 3A and 3B in regulating platelet and cardiac function using subtype-selective knockout mice. *Cell Signal* 19:1765–1771
- Thompson PE, Manganiello V, Degerman E (2007) Re-discovering PDE3 inhibitors - new opportunities for a long neglected target. *Curr Top Med Chem* 7:421–436
- Thomson NC, Spears M (2005) The influence of smoking on the treatment response in patients with asthma. *Curr Opin Allergy Clin Immunol* 5:57–63
- Thomson NC, Chaudhuri R, Livingston E (2004) Asthma and cigarette smoking. *Eur Respir J* 24:822–833
- Thomson NC, Shepherd M, Spears M, Chaudhuri R (2006) Corticosteroid insensitivity in smokers with asthma: clinical evidence, mechanisms, and management. *Treat Respir Med* 5: 467–481
- Timms RM, Khaja FU, Williams GW (1985) Hemodynamic response to oxygen therapy in chronic obstructive pulmonary disease. *Ann Intern Med* 102:29–36
- Tiwari S, Dong H, Kim EJ, Weintraub L, Epstein PM, Lerner A (2005) Type 4 cAMP phosphodiesterase (PDE4) inhibitors augment glucocorticoid-mediated apoptosis in B cell chronic lymphocytic leukemia (B-CLL) in the absence of exogenous adenylyl cyclase stimulation. *Biochem Pharmacol* 69:473–483
- Torphy TJ (1998) Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. *Am J Respir Crit Care Med* 157:351–370
- Torphy TJ, Zhou HL, Burman M, Huang LB (1991) Role of cyclic nucleotide phosphodiesterase isozymes in intact canine trachealis. *Mol Pharmacol* 39:376–384
- Torphy TJ, Udem BJ, Cieslinski LB, Luttmann MA, Reeves ML, Hay DW (1993) Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle. *J Pharmacol Exp Ther* 265:1213–1223
- Ukena D, Harnest U, Sakalauskas R, Magyar P, Vetter N, Steffen H, Leichtl S, Rathgeb F, Keller A, Steinijans VW (1997) Comparison of addition of theophylline to inhaled steroid with doubling of the dose of inhaled steroid in asthma. *Eur Respir J* 10:2754–2760
- Vergne F, Bernardelli P, Chevalier E (2005) PDE7 inhibitors: chemistry and potential therapeutic utilities. *Ann Rev Med Chem* 40:227–241
- Vijaykrishnan L, Rudra S, Eapen MS, Dastidar S, Ray A (2007) Small-molecule inhibitors of PDE-IV and -VII in the treatment of respiratory diseases and chronic inflammation. *Expert Opin Investig Drugs* 16:1585–1599
- Wang Z, Malone MH, Thomenius MJ, Zhong F, Xu F, Distelhorst CW (2003) Dexamethasone-induced gene 2 (dig2) is a novel pro-survival stress gene induced rapidly by diverse apoptotic signals. *J Biol Chem* 278:27053–27058
- Wenzel S (2003) Pathology of difficult asthma. *Paediatr Respir Rev* 4:306–311

- Wharton J, Strange JW, Moller GM, Growcott EJ, Ren X, Franklyn AP, Phillips SC, Wilkins MR (2005) Antiproliferative effects of phosphodiesterase type 5 inhibition in human pulmonary artery cells. *Am J Respir Crit Care Med* 172:105–113
- Wiggs BR, Moreno R, Hogg JC, Hilliam C, Pare PD (1990) A model of the mechanics of airway narrowing. *J Appl Physiol* 69:849–860
- Wilkens H, Guth A, Konig J, Forestier N, Cremers B, Hennen B, Bohm M, Sybrecht GW (2001) Effect of inhaled iloprost plus oral sildenafil in patients with primary pulmonary hypertension. *Circulation* 104:1218–1222
- Wilsmhurst PT, Webb-Peploe MM (1983) Side effects of amrinone therapy. *Br Heart J* 49:447–451
- Wilson SM, Shen P, Rider CF, Traves SL, Proud D, Newton R, Giembycz MA (2009) Selective prostacyclin receptor agonism augments glucocorticoid-induced gene expression in human bronchial epithelial cells. *J Immunol* 183:6788–6799
- Woodrow MD, Ballantine SP, Barker MD, Clarke BJ, Dawson J, Dean TW, Delves CJ, Evans B, Gough SL, Guntrip SB, Holman S, Holmes DS, Kranz M, Lindvaal MK, Lucas FS, Neu M, Ranshaw LE, Solanke YE, Somers DO, Ward P, Wiseman JO (2009) Quinolines as a novel structural class of potent and selective PDE4 inhibitors. Optimisation for inhaled administration. *Bioorg Med Chem Lett* 19:5261–5265
- Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD, Carter R, Wong HH, Cadbury PS, Fahy JV (2004) Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. *Am J Respir Crit Care Med* 169:1001–1006
- Wright LC, Seybold J, Robichaud A, Adcock IM, Barnes PJ (1998) Phosphodiesterase expression in human epithelial cells. *Am J Physiol* 275:L694–L700
- Yamamoto S, Sugahara S, Ikeda K, Shimizu Y (2006a) Pharmacological profile of a novel phosphodiesterase 7A and -4 dual inhibitor, YM-393059, on acute and chronic inflammation models. *Eur J Pharmacol* 550:166–172
- Yamamoto S, Sugahara S, Naito R, Ichikawa A, Ikeda K, Yamada T, Shimizu Y (2006b) The effects of a novel phosphodiesterase 7A and -4 dual inhibitor, YM-393059, on T-cell-related cytokine production in vitro and in vivo. *Eur J Pharmacol* 541:106–114
- Yamamoto S, Sugahara S, Ikeda K, Shimizu Y (2007a) Amelioration of collagen-induced arthritis in mice by a novel phosphodiesterase 7 and 4 dual inhibitor, YM-393059. *Eur J Pharmacol* 559:219–226
- Yamamoto T, Shimano H, Inoue N, Nakagawa Y, Matsuzaka T, Takahashi A, Yahagi N, Sone H, Suzuki H, Toyoshima H, Yamada N (2007b) Protein kinase A suppresses sterol regulatory element-binding protein-1C expression via phosphorylation of liver X receptor in the liver. *J Biol Chem* 282:11687–11695
- Yamashita K, Kobayashi S, Okada K, Tsunematsu T (1990) Increased external carotid artery blood flow in headache patients induced by cilostazol. Preliminary communication. *Arzneimittelforschung* 40:587–588
- Yang G, McIntyre KW, Townsend RM, Shen HH, Pitts WJ, Dodd JH, Nadler SG, McKinnon M, Watson AJ (2003) Phosphodiesterase 7A-deficient mice have functional T cells. *J Immunol* 171:6414–6420
- Ye RD (2000) β -Adrenergic agonists regulate NF- κ B activation through multiple mechanisms. *Am J Physiol Lung Cell Mol Physiol* 279:L615–L617
- Yu SM, Tsai SY, Kuo SC, Ou JT (1996) Inhibition of platelet function by A02131-1, a novel inhibitor of cGMP-specific phosphodiesterase, in vitro and in vivo. *Blood* 87:3758–3767
- Zhang L, Insel PA (2004) The pro-apoptotic protein Bim is a convergence point for cAMP/protein kinase A- and glucocorticoid-promoted apoptosis of lymphoid cells. *J Biol Chem* 279:20858–20865
- Zhang YK, Plattner JJ, Akama T, Baker SJ, Hernandez VS, Sanders V, Freund Y, Kimura R, Bu W, Hold KM, Lu XS (2010) Design and synthesis of boron-containing PDE4 inhibitors using soft-drug strategy for potential dermatologic anti-inflammatory application. *Bioorg Med Chem Lett* 20:2270–2274

Phosphodiesterases in the Central Nervous System: Implications in Mood and Cognitive Disorders

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Abstract Cyclic nucleotide phosphodiesterases (PDEs) are a superfamily of enzymes that are involved in the regulation of the intracellular second messengers cyclic AMP (cAMP) and cyclic GMP (cGMP) by controlling their rates of hydrolysis. There are 11 different PDE families and each family typically has multiple

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isoforms and splice variants. The PDEs differ in their structures, distribution, modes of regulation, and sensitivity to inhibitors. Since PDEs have been shown to play distinct roles in processes of emotion and related learning and memory processes, selective PDE inhibitors, by preventing the breakdown of cAMP and/or cGMP, modulate mood and related cognitive activity. This review discusses the current state and future development in the burgeoning field of PDEs in the central nervous system. It is becoming increasingly clear that PDE inhibitors have therapeutic potential for the treatment of neuropsychiatric disorders involving disturbances of mood, emotion, and cognition.

Keywords Anxiety · cAMP · cGMP · Cognition · Depression · Phosphodiesterases · Schizophrenia

1 Introduction

Phosphodiesterases (PDEs) are the only known enzymes that catalyze the hydrolysis of cyclic AMP (cAMP) and cyclic GMP (cGMP) and thus are integral to the regulation of cyclic nucleotide signaling and cellular communication. PDEs are encoded by 21 genes and are functionally separated into 11 families, which are differently expressed in various tissues throughout the body, including the brain (Conti and Beavo 2007; Lugnier 2006). PDE families are divided into three categories based on their substrate specificity: PDE 4, 7, and 8 hydrolyze cAMP; PDE 5, 6, and 9 hydrolyze cGMP; and PDE 1, 2, 3, 10, and 11 are dual substrate enzymes and hydrolyze both cAMP and cGMP. The PDEs can also be classified by the mechanisms regulating their activity, such as regulation by cGMP or Ca^{2+} /calmodulin, or their N-terminal regulatory domains, such as specific protein-binding regions, phosphorylation sites, and GAF domains. Current studies indicate that there are more than 100 different protein products transcribed from the PDEs genes because of alternative transcriptional start sites and alternative splicing of gene products (Lugnier 2006; Bender and Beavo 2006; Menniti et al. 2006). The multiplicity of the PDE gene family and the complex expression patterns of the different PDEs have raised questions regarding the physiological and pathological relevance of the multiple isoforms of these enzymes. Various PDEs have been shown to be involved in intracellular signaling pathways associated with neuropsychiatric disorders or their treatment (O'Donnell and Zhang 2004; Kelly and Brandon 2009; Millar et al. 2007; Schmidt 2010; Siuciak 2008; Zhang 2009). Inhibitors of at least seven PDEs families have been implicated in behavioral changes related to cognition, depression, and anxiety, namely those for PDE 1, 2, 4, 5, 9, 10, and 11. In this review, we provide an overview of the PDE gene superfamily and the effect of specific PDE inhibitors on intracellular signaling with a particular focus on mood and cognitive disorders.

2 Distribution of PDE Isoforms in the Brain

Studies indicate a high distribution of PDEs in the frontal cortex, hippocampus, amygdala, olfactory bulb, striatum, hypothalamus, and cerebellum, besides the expression in numerous other body structures, which are important in modulating emotion, learning, and memory. Since PDEs are involved in the regulation of second messenger signaling, their inhibition in the brain has provided valuable insights into the biological mechanisms that underlie central nervous system (CNS) disorders and the discovery of mood and cognitive modulators.

PDE1 consists of three different subtypes, 1A, 1B, and 1C, which can be found in the human brain at the level of hippocampus and cerebral cortex, suggesting that this enzyme may play a crucial role in memory formation and storage (Deshmukh et al. 2009). The high concentrations of PDE1A and PDE1B mRNA expression in olfactory bulb and striatum indicate their potential function in depression, schizophrenia, and other mental and cognitive illnesses. PDE1C is expressed exclusively in the olfactory cilia together with adenylyl cyclase III, which are both Ca^{2+} -regulated enzymes and form a point of crosstalk for integrating calcium and cAMP signaling in these neurons (Juilfs et al. 1997).

PDE2 is highly expressed in brain limbic structures and adrenal gland (Boess et al. 2004; Nikolaev et al. 2005). The enriched PDE2 expression in the hippocampus, cerebral cortex, amygdala, hypothalamus, pituitary, and adrenal cortex suggests that it may be involved in negative feedback inhibition of the limbic–hypothalamus–pituitary–adrenal (limbic–HPA) axis in emotion, and learning and memory processes.

PDE3 is expressed at relatively constant and low levels throughout the brain (Bolger et al. 1994).

A high distribution of PDE4 in the hippocampus, frontal cortex, olfactory bulb, and cerebellum has been reported (Kaulen et al. 1989; Zhan et al. 2003; Dlaboga et al. 2006; McPhee et al. 2001). Four PDE4 genes (PDE4A, 4B, 4C, and 4D), encoding at least 25 splice variants, have been identified (Conti et al. 2003; Houslay et al. 2007; Houslay 2010). Long-form variants contain two conserved regions called Upstream Conserved Region 1 (UCR1) and 2 (UCR2), while short isoforms lack UCR1 (Bolger et al. 1993). Long isoforms contain a site within UCR1 that confers stimulatory phosphorylation by protein kinase A (Sette et al. 1994; MacKenzie et al. 2002). PDE4 subtypes appear to be involved in different central functions based on their patterns of distribution in the brain (Cherry and Davis 1999; Engels et al. 1995; McPhee et al. 2001; Miró et al. 2002; Pérez-Torres et al. 2000; Zhang 2009). PDE4A and PDE4D are highly expressed in the cerebral cortex, olfactory bulb, hippocampal formation, and brainstem. PDE4B is highly expressed in the amygdala, striatum, and hypothalamus, suggesting its possible role in the mediation or treatment of anxiety and schizophrenia. The side effects of

PDE4 inhibitors of nausea and emesis may be related to the high expression of PDE4, particularly the PDE4D subtype, in the area postrema (Mori et al. 2010).

In the brain, PDE5 is mainly distributed in cerebral cortex, pyramidal cells of the hippocampus, basal ganglia, and most abundantly cerebellum (van Staveren et al. 2003; Kleppisch 2009; Garthwaite and Boulton 1995).

PDE6 is confined to the retina and pineal gland and appears to play no direct role in mediating psychopharmacological effects on behavior (Bender and Beavo 2006).

High PDE7A mRNA signal intensities are detected in the hippocampus, olfactory bulb and tubercle, and brainstem nuclei (Miro et al. 2001), while PDE7B is present in both neuronal and nonneuronal cell populations in multiple brain regions, such as cerebellum, striatal complex, dentate gyrus of hippocampus, and in several thalamic nuclei (Pérez-Torres et al. 2003; Sasaki et al. 2002).

PDE8A transcripts are rich in peripheral tissues, while PDE8B transcripts are found in all brain regions of rats other than cerebellum and have been shown to exist in neurons (Kobayashi et al. 2003).

PDE9 signals are strong in cerebellar Purkinje cells, cerebral cortex, and hippocampus (van Staveren et al. 2001, 2003). Neuronal PDE9 expression has been detected in the cerebellum, cerebral cortex, caudate-putamen, and hippocampus.

The dual-specificity PDE10 family is encoded by one gene, PDE10A, which has emerged as a key therapeutic target for treatment of schizophrenia (Siuciak 2008; Kelly and Brandon 2009). It is interesting that the apparent function of PDE10A relates to its unique distribution in the brain, which is very high in the caudate-putamen, nucleus accumbens, and olfactory tubercle, with minimal expression in cortex, hippocampus, and cerebellum (Kelly et al. 2010).

The PDE11 family is the most recently described PDE family and also contains one gene, PDE11A; four splice variants have been described (Loughney et al. 2005). PDE11A mRNA and protein are largely restricted to the hippocampus, with two- to threefold enrichment in the ventral vs. dorsal hippocampus, equal distribution between cytosolic and membrane fractions, and increasing levels of protein expression from postnatal day 7 through adulthood (Kelly et al. 2010).

The relative distribution patterns of the different PDE isoforms in the brain are shown in Table 1. The differential expression patterns of PDEs in the brain and their roles in controlling neuronal activity indicate that targeted PDE inhibition may result in therapeutic effects. However, some side effects of PDE inhibitors likely are also associated with the complexity of PDEs expression patterns across brain areas.

3 Roles of PDEs in Mood and Cognitive Disorders

The complex functions of PDEs in cell–cell communication and signal transduction are only recently being recognized, although PDEs were described soon after the discovery of cAMP. A PDE gene organization with a large number of PDE splice variants serves to fine-tune cyclic nucleotide concentrations and compartmentalization

Table 1 Distribution of PDE isoforms in the brain

Isoform	Hippocampus	Cortex	Cerebellum	Olfactory bulb	Striatum	Amygdala	Hypothalamus	References
PDE1A	+	+	+	+	+	-	-	Deshmukh et al. (2009)
PDE1B	+	+	-	+	+	+	-	
PDE1C	+	+	+	-	-	+	-	
PDE2A	+	+	-	+	+	+	+	Boess et al. (2004), Nikolaev et al. (2005)
PDE3A	+	+	-	+	+	+	+	Bolger et al. (1994)
PDE3B	+	+	-	+	+	-	-	
PDE4A	+	+	+	+	+	+	+	Zhan et al. (2003), Dlaboga et al. (2006)
PDE4B	+	+	+	-	+	-	+	
PDE4C	-	-	-	-	-	-	-	
PDE4D	+	+	+	-	+	-	+	
PDE5A	+	+	+	-	-	-	-	van Staveren et al. (2003)
PDE6A	-	-	-	-	-	-	-	Bender and Beavo (2006)
PDE6B	-	-	-	-	-	-	-	
PDE6C	-	-	-	-	-	-	-	
PDE6D	-	-	-	-	-	-	-	
PDE7A	+	+	+	+	+	-	-	Pérez-Torres et al. (2003)
PDE7B	+	+	+	-	+	-	-	
PDE8A	-	-	-	-	-	-	-	Kobayashi et al. (2003)
PDE8B	+	+	+	+	+	-	-	
PDE9A	+	+	+	+	+	+	+	van Staveren et al. (2001, 2003)
PDE10A	+	+	+	-	+	-	-	Kelly et al. (2010)
PDE11A	+	-	-	-	-	-	-	Kelly et al. (2010)

and contributes to specificity in intracellular signaling (Conti and Beavo 2007; Houslay 2010).

3.1 PDE1

PDE1 family members are considered dual-substrate enzymes involved in the regulation of both cGMP and cAMP through their degradation (Bender and Beavo 2006). In vitro, PDE1A and PDE1B isozymes hydrolyze cGMP with much higher affinity than cAMP, while the PDE1C isozyme hydrolyzes cAMP and cGMP with equally high affinity. In vivo, several studies have shown that PDE1A and PDE1B primarily regulate cGMP (Nagel et al. 2006; Bender and Beavo 2006; Miller et al. 2009), while PDE1C regulates intracellular cAMP levels in various cell types including neurons (Rybalkin et al. 2002; Han et al. 1999; Dunkern and Hatzelmann 2007).

PDE1 inhibition has been reported to cause cerebral artery dilation, which can affect CNS function by elevation of cGMP levels (Kruuse et al. 2001). The PDE1 inhibitor vinpocetine facilitates long-term potentiation (Molnar and Gaal 1992), enhances the structural dynamics of dendritic spines, and improves aversion-related memory retrieval (DeNoble 1987). A recent study indicates that vinpocetine treatment of streptozotocin-infused rats improves memory consolidation as evidenced by increased time spent in the target quadrant in the Morris water maze test (Deshmukh et al. 2009). Most recently, Filgueiras and colleagues (2010) reported that vinpocetine improves learning and memory deficits in rats exposed to alcohol.

3.2 PDE2

PDE2 is a 105-kDa homodimer that exists in particulate and soluble forms (Zaccolo and Movsesian 2007). It belongs to a family of proteins that regulates the intracellular levels of both cGMP and cAMP. Although cGMP is the preferred substrate for this enzyme, PDE2 hydrolyzes both cGMP and cAMP with positively cooperative kinetics (Wu et al. 2004). Therefore, the biological function of PDE2 may, to some degree, involve interaction between cAMP- and cGMP-mediated signaling mechanisms (Hajjhussein et al. 2007).

The distribution of PDE2 in limbic structures, such as hippocampus and amygdala, may suggest a role in emotion and memory operations, such as the formation of stable declarative (or explicit) memory in humans and spatial (or relational/contextual) memory in rodents. PDE2 is also found in a subset of olfactory neurons, where its inhibition increases cGMP, stimulates cyclic nucleotide-gated channels to open, and initiates a depolarizing response (Meyer et al. 2000). Neuronal projections from the olfactory bulbs to the limbic system have a major influence on emotional behaviors involved in depression and anxiety. In addition, depressive symptoms

correlate negatively with olfactory sensitivity in healthy participants, which is confirmed by patients with major depressive disorder (Pollatos et al. 2007; Pause et al. 2001). PDE2 is also found in pituitary and adrenal cortex (Blokland et al. 2006). Menniti and coworkers (2006) suggested that PDE2 influences cAMP metabolism in the pituitary and adrenal cortex, while it regulates cGMP signaling in the cerebral cortex and hippocampus. In primary neuronal cultures, inhibition of PDE2 with the selective inhibitor EHNA enhances *N*-methyl-D-aspartate (NMDA) receptor-mediated cGMP levels, but cAMP is unaffected (Suvarna and O'Donnell 2002). The highly selective PDE2 inhibitor BAY 60-7550, which unlike EHNA does not inhibit adenosine deaminase, increases cGMP levels in the presence of NMDA or guanylyl cyclase activators. Although BAY 60-7550 also increases NMDA receptor-induced cAMP levels in cultured neurons, the magnitude of this effect is markedly less than the increase in cGMP (Masood et al. 2009). Several reports suggest cyclic nucleotide pathways can crosstalk to modulate each other's synthesis, degradation, and actions (Surapisitchat et al. 2007; Aizawa et al. 2003). It is possible that the high level of cGMP acts by binding to an allosteric site on PDE2 that activates PDE2-mediated breakdown of cAMP in neurons (Pelligrino and Wang 1998).

BAY 60-7550 has been observed to fully reverse the working memory deficit induced by the NMDA receptor antagonist MK-801. It improves the performance of rats in social and object recognition memory tasks, and reverses MK801-induced deficits in spontaneous alternation in a T-maze test (Boess et al. 2004). BAY 60-7550 also improves short-term object recognition performance after an acute tryptophan depletion-induced memory deficit (van Donkelaar et al. 2008). These results are in agreement with a report suggesting that BAY 60-7550 improves acquisition and consolidation in the object recognition task in rats with age-related cognitive impairments (Domek-Łopacińska and Strosznajder 2008). It is known that there is a lower cGMP concentration in the aged brain and an alteration in the activity of cGMP-hydrolyzing PDEs. Therefore, the mechanism behind the beneficial effects of PDE2 inhibitors on cognition defects may be related to stimulating the cGMP-regulated signal cascade and subsequently enhancing neuronal plasticity (Boess et al. 2004).

PDE2 inhibition is able to attenuate oxidative stress-induced anxiety in mice, as well as anxiety-like behaviors produced by other means (Masood et al. 2008, 2009). The anxiolytic effects of two PDE2 inhibitors Bay 60-7550 and ND7001 are observed in the elevated plus-maze, hole-board, and open-field tests in both stressed and nonstressed mice; antagonism experiments suggest these behavioral effects result from increased cGMP signaling (Figs. 1 and 2). The effects on oxidative stress are confirmed using primary neuronal cultures, which show that Bay 60-7550 and ND7001 reverse oxidant-induced increases in reactive oxygen species and improve the total antioxidant capacity in cerebral cortical neurons. These various behavioral and neurochemical data suggest that PDE2 may be a useful pharmacological target for treatment of neurodegenerative and psychiatric diseases that involve increased oxidative stress.

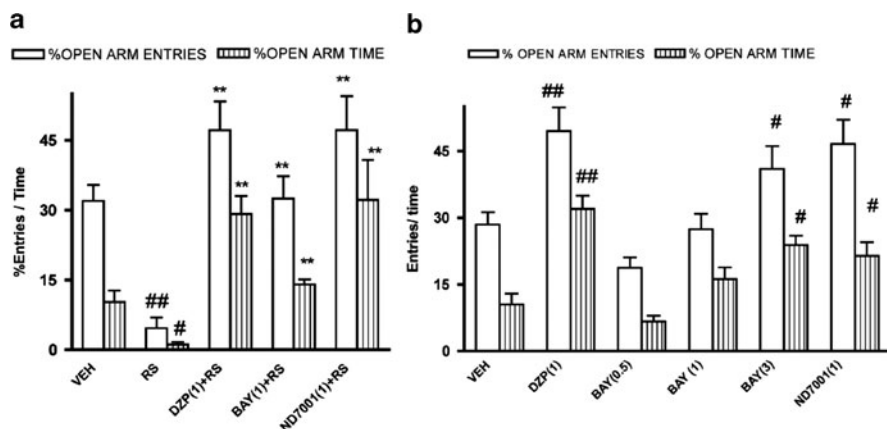


Fig. 1 Effects of the PDE2 inhibitors Bay 60-7550 (BAY) and ND7001 on behavior in the elevated plus-maze in stressed (a) and nonstressed (b) mice. Increases in percentages open-arm entries and time indicate an anxiolytic effect, whereas decreases in these measures indicate an anxiogenic effect. Values are expressed as mean \pm SEM. ($n = 6-8$). # $p < 0.05$ and ## $p < 0.01$ compared with vehicle (VEH). ** $p < 0.01$ compared with restraint stress (RS). *DZP* diazepam. Doses shown parenthetically are milligrams per kilogram i.p. Reprinted with permission from (Masood et al. 2009)

3.3 PDE3

PDE3 was originally characterized by its high affinity for both cAMP and cGMP, but subsequent *in vivo* studies suggested that the hydrolysis of cAMP is inhibited by cGMP. This finding is supported by the data from intact cells containing PDE3 (Maurice and Haslam 1990). Two PDE3 isoforms, PDE3A and PDE3B, have different subcellular locations due to their different N-terminal regions, but have similar kinetic properties since they have amino acid identity over their catalytic sites (Bender and Beavo 2006; Pyne et al. 1986). The activity of PDE3 is modulated in several cell types by phosphorylation in response to hormonal stimulation by prostaglandins, epinephrine, and insulin (Murata et al. 2009; Zaccolo and Movsesian 2007; Thompson et al. 2007; Palmer et al. 2007). PDE3 specific inhibitors including amrinone, trequinsin, and cilostazol, have been identified, but they do not distinguish between PDE3A and PDE3B (Thompson et al. 2007).

PDE3 inhibitor effects are related to preventing platelet aggregation and regulating myocardial contractility and smooth muscular vasodilation. One of the inhibitors, cilostazol, is reported to increase insulin-like growth factor I (IGF-I) and cAMP levels in the hippocampus (Zhao et al. 2010). It is speculated that this results in stimulation of sensory neurons resulting in improved cognitive function in mice (Zhao et al. 2010). Further studies are needed to confirm the effects of PDE3 inhibitors in the CNS, particularly in the modulation of learning and memory.

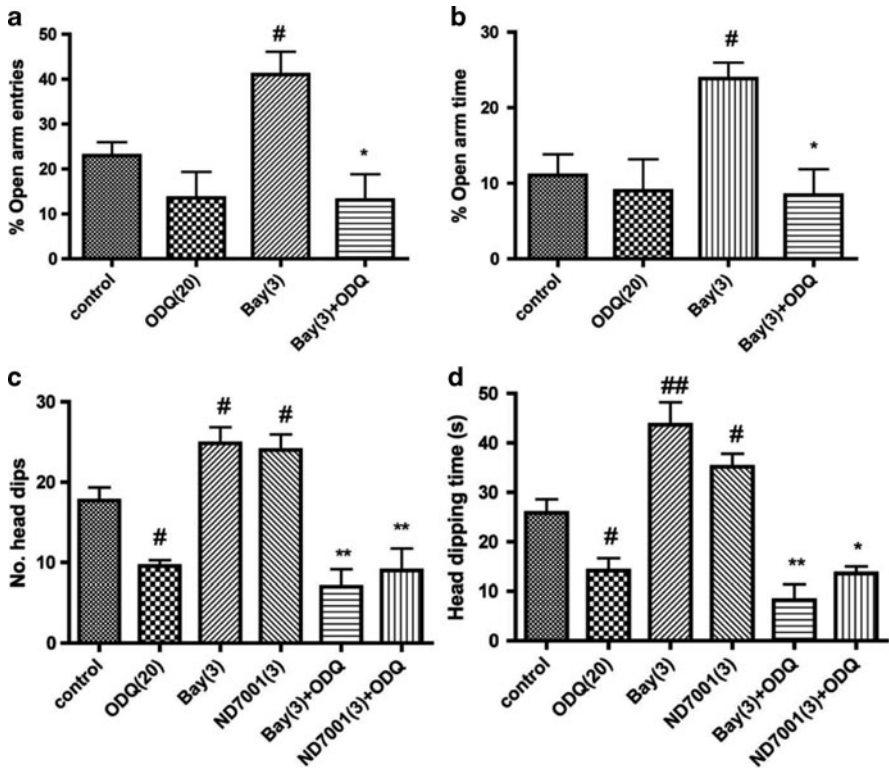


Fig. 2 Antagonistic effects of the guanylyl cyclase inhibitor ODQ (20 mg/kg) on the anxiolytic effects of Bay 60-7550 (Bay) in the elevated plus-maze (**a**, **b**) and both Bay 60-7550 and ND7001 in the hole-board tests (**c** and **d**). Values are expressed as means \pm SEM ($n = 6-8$). # $p < 0.05$; ## $p < 0.01$ compared to control; * $p < 0.05$; ** $p < 0.01$ compared to Bay 60-7550 and ND7001. Doses shown parenthetically are mg/kg, i.p. Reprinted with permission from (Masood et al. 2009)

3.4 PDE4

PDE4 consists of a large family of isoforms encoded by four genes that specifically hydrolyze cAMP with high affinity and are not sensitive to either cGMP or Ca^{2+} /calmodulin (Conti et al. 2003; Houslay et al. 2007; Houslay 2010). Individual isoforms are thought to have specific functional roles in cells by virtue of association with partner proteins that target them to functionally relevant sites in the cell (Houslay 2010).

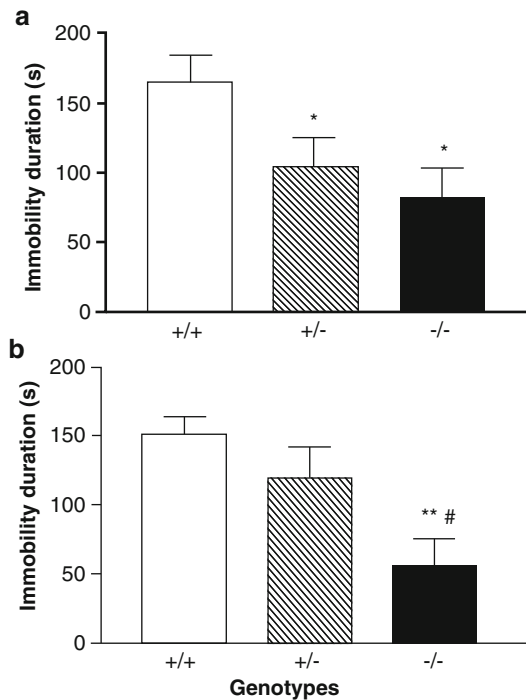
Interest linking PDE4 to cognitive effects stemmed from the pioneering work of Wachtel (1982), who described the discovery of a selective PDE4 inhibitor rolipram and its antidepressant effects. PDE4 has since been implicated in various disorders such as depression, anxiety, and inflammation-related disorders (Barnette and Underwood 2000; Houslay 2001; Houslay et al. 2005; O'Donnell and Zhang 2004; Zhang 2009). Recent studies have extended the therapeutic potential of PDE4

inhibitors to a variety of other CNS diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, attention-deficit hyperactivity disorder, schizophrenia, stroke, autism, and tardive dyskinesia (Cheng et al. 2010; Titus et al. 2008; Millar et al. 2007).

PDE4A and PDE4D may be important in the mediation of antidepressant activity and memory, while PDE4B is more likely involved in dopamine-associated and stress-related processes. This is supported by studies showing that PDE4D is important in antidepressant-like behavior, whereas PDE4B appears to be involved in signaling pathways important for schizophrenia and anxiety (Zhang et al. 2002a, b, 2008; Millar et al. 2005; Siuciak et al. 2007) (Figs. 3 and 4).

Rolipram is a selective inhibitor of PDE4 and increases intracellular cAMP by inhibiting its hydrolysis (Houslay et al. 2005). Research investigating PDE4 function using rolipram suggests that, given peripherally, rolipram elevates cAMP levels in the brain and enhances retention in passive avoidance tasks in mice, which is consistent with the concept that cAMP is related to neural processes that underlie learning and memory (Zhang et al. 2000, 2005; Rutten et al. 2006). Subsequent studies have shown the positive effects of PDE4 inhibition on other types of memory, such as working and reference memory and contextual fear memory (Gong et al. 2004; Zhang and O'Donnell 2000). Working memory refers to processes encoding items related to the temporal/personal context of a single event or a test situation, while reference memory encodes context-independent

Fig. 3 PDE4D^{-/-} mice exhibited antidepressant-like behavior in the tail-suspension and forced-swim tests. **(a)** The immobility duration in the tail suspension test. **(b)** The immobility duration in the forced swim test in the PDE4D^{+/+}, PDE4D^{+/-} and PDE4D^{-/-} mice. Values shown are mean \pm SEM; * $p < 0.05$, ** $p < 0.01$ vs. PDE4D^{+/+}; # $p < 0.05$ vs. PDE4D^{+/-} ($n = 7$). Reprinted with permission from (Zhang et al. 2002a)



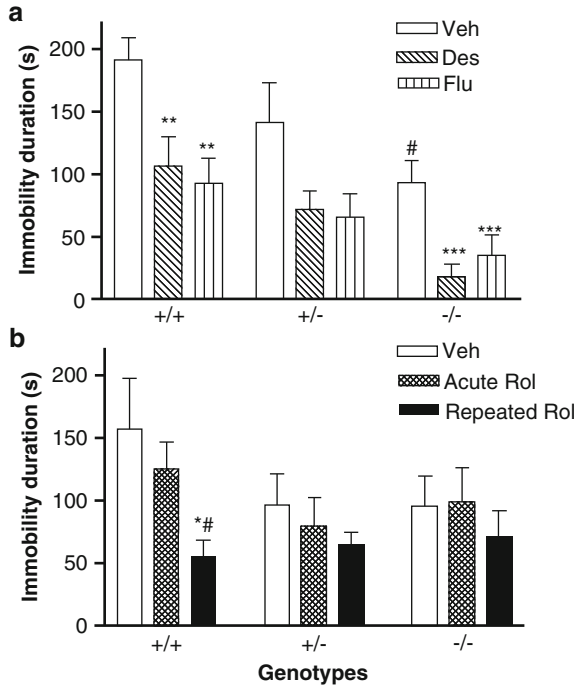


Fig. 4 Effects of antidepressants on immobility duration in the forced swim test in the PDE4D+/+, PDE4D+/-, and PDE4D-/- mice. **(a)** The effect of desipramine (Des) or fluoxetine (Flu). **(b)** The effect of acute or repeated treatment with rolipram (Rol, 0.5 mg/kg). All the drugs or vehicles [Veh; saline in **(a)** and 10% DMSO in **(b)**] were administered 30 min before the test. For the test involving repeated treatment with rolipram, the drug was administered once daily for 8 days; the forced swim test was conducted 30 min after the last injection (i.e., on day eight). Values shown are mean \pm SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. corresponding genotype administered vehicle (Veh); # $p < 0.05$ vs. PDE4D+/+ following the same treatment ($n = 5-7$). Reprinted with permission from (Zhang et al. 2002a)

rules and procedures that are specific to a given situation and which remain valid each time the situation is encountered (Olton 1983; Cassel et al. 1998). Scopolamine, a muscarinic cholinergic receptor antagonist, has been used to induce impairment of working and reference memory. Rolipram reverses scopolamine-induced deficits in both working memory and reference memory in the radial-arm maze test (Zhang and O'Donnell 2000).

Contextual fear memory can be tested in a paradigm of fear renewal that is a model to study the interplay of context and memory retrieval. In this paradigm, a change of context after extinction results in robust return of the conditioned fear response. The renewed expression of fear demonstrates that extinction memory is gated by contextual information (Bouton et al. 2006). Recent studies suggest that rolipram enhances long-term memory formation in the conditioned fear response test (Mueller et al. 2010). Understanding the neurobehavioral mechanisms

underlying the expression and reduction of fear may help improve pharmacologic treatments for emotion-related memory disorders.

In addition, PDE4 inhibitors reverse working memory deficits caused by the NMDA receptor antagonist (MK-801), MEK inhibition, electroconvulsive shock (ECS), and age- or microsphere embolism-induced cerebral ischemia in the radial-arm maze and the three-panel runway task (Zhang et al. 2004, 2005; Reneerkens et al. 2009). Another study has shown that two novel PDE4 inhibitors, MEM1018 and MEM1091, improve memory processes in radial-arm maze and passive avoidance tests (Zhang et al. 2005). Consistent with these behavioral data, the PDE4 inhibitors produce parallel and consistent effects on cAMP concentrations in cultured neurons that are pharmacologically altered through inhibition of NMDA receptors or MEK (Suvarna and O'Donnell 2002; Zhang et al. 2004).

It has been reported that the effects of rolipram infusion into prefrontal cortex on working memory in young and aged animals are different. It improves working memory in young rhesus monkeys in a delayed responding task in a T-maze test, but has a negative effect on working memory in aged monkeys in this task (Ramos et al. 2003, 2006). The underlying mechanism may involve aged animals with prefrontal cortical cognitive deficits being more sensitive to both the impairing effects of PKA activation and the enhancing effects of PKA inhibition (Ramos et al. 2006). Molecular analyses show that the basal PKA levels (i.e., regulatory or catalytic subunit immunoreactivity) are changed in aged prefrontal cortex, but the levels in hippocampus are not affected by aging. Moreover, CRE binding is significantly higher in the aged prefrontal cortex but not in the hippocampus (Ramos et al. 2003). It is known that the increase in CRE binding may indirectly reflect increased PKA activity since activation of this pathway has been shown to upregulate CRE-mediated gene expression, including the cAMP responsive element binding protein (CREB) gene itself (Coven et al. 1998). Although activation of the cAMP/PKA pathway in the hippocampus has been proposed as a mechanism for improving age-related cognitive impairment, the deficits in prefrontal cortex function with advancing age have received increasing attention, particularly in human and nonhuman primates. The prefrontal cortex is engaged during the retrieval and encoding of memories and is critical for inhibition of proactive interference, for protecting memories and thoughts from distraction, and for allowing the planning and organization of behavior (Bunge et al. 2001; Lepage et al. 2000). Consistent with this, PDE4 is highly expressed in the prefrontal cortex and hippocampus (Reneerkens et al. 2009), which makes a predominant contribution to cAMP hydrolysis in the brain and plays an important role in emotion and memory processing. Recent, exciting data suggest that activation of cAMP-dependent protein kinase A is crucial for long-term potentiation and long-term depression (LTD) of synaptic transmission in the hippocampus, phenomena that are thought to be involved in memory formation (Ster et al. 2009; Whitaker and Wei 2009). The studies reported the role of an alternative target of cAMP, the exchange protein factors directly activated by cAMP (Epac1 and Epac2) that belong to a large family of guanine exchange factor proteins that catalyze the activation of small G-proteins, particularly Rap1 and Rap2 (Ster et al. 2009; Bos 2006). The results showed

that pharmacological activation of Epac by the selective agonist 8-(4-chlorophenylthio)-2-*O*-methyl-cAMP (8-pCPT) induces LTD in the CA1 region. The 8-pCPT-induced LTD was blocked by the Epac signaling inhibitor, Rap-1 antagonist and p38 mitogen-activated protein kinase (P38-MAPK) inhibitor. This indicates a direct involvement of Epac in LTD. In addition, the activation of Epac2 and Rap guanine-nucleotide exchange factor (GEF) induces synapse remodeling and depression in cultured rat cortical neurons, which contributes to neural circuit development and plasticity of brain circuits throughout life (Woolfrey et al. 2009; Holtmaat et al. 2005; Oray et al. 2004).

PKA activation affects learning and memory through downstream phosphorylation events, gene regulation, and synapse remodeling (Nguyen and Woo 2003; Woolfrey et al. 2009). Infusions of the PKA activator Sp-cAMPs or PDE4 inhibitor rolipram into the aged brain impairs or improves working memory performance under conditions that require hippocampal-prefrontal cortex interactions (Taylor et al. 1999; Birnbaum et al. 1999; Ramos et al. 2006). Additional research is necessary to identify the role of PDE4 in age-related behavioral changes, as well as mechanisms that regulate PKA activity.

The deposition of beta-amyloid in the cerebral cortex and hippocampus is considered as an early and critical event in the pathogenesis of age-related mood and cognitive disorders associated with Alzheimer's disease. Recent studies have shown that repeated treatment with rolipram reverses memory deficits in the water maze and passive avoidance tasks induced by Abeta 25-35 or Abeta 1-40 (Cheng et al. 2010). The cognition enhancement by rolipram appears to be attributed to rolipram-induced increases in cAMP/CREB signaling in the hippocampus. These findings are in agreement with rolipram-induced reversal of memory deficits in APP/PS1 transgenic mice (Gong et al. 2004). However, the involvement of particular PDE4 subtypes in mediating cognition has yet to be elucidated.

Schizophrenia has a complex genetic underpinning, and variations in a number of candidate genes have been identified that confer risk of developing the disorder (Fatemi et al. 2008). The PDE4B gene is located at 1p31 that is a susceptibility region for schizophrenia (Numata et al. 2008; Pickard et al. 2007). Significant changes between wild-type and PDE4B knockout mice have been found in several behavioral tasks, e.g., decreases in baseline levels of monoamines and their metabolites in the striatum, decreased baseline motor activity, and an exaggerated locomotor response to amphetamine (Siuciak et al. 2008). These data suggest a role for PDE4B in neuropsychiatric diseases and striatal function. Studies in our laboratory (Zhang et al. 2008) have found that PDE4B knockout mice display anxiogenic-like behaviors, as evidenced by decreased head-dips and time spent in head-dipping in the hole board test, reduced transitions and time on the light side in the light-dark transition test, and decreased initial exploration and rears in the open-field test; inconsistent effects were found in models of antidepressant sensitivity. Neurochemical studies (Takahashi et al. 1999) suggest that PDE4B in the frontal cortex and nucleus accumbens, but not the hippocampus, is upregulated by chronic antidepressant administration. The reports from clinical studies further support the genetic linkage of PDE4B with schizophrenia in some patients (Pickard et al. 2007;

Numata et al. 2008; Fatemi et al. 2008). Another important finding is the interaction of endogenous disrupted-in-schizophrenia 1 (DISC1) and PDE4B in schizophrenia (Millar et al. 2005; Siuciak et al. 2008; Clapcote et al. 2007). DISC1 is a genetic susceptibility factor for schizophrenia and is a multifunctional scaffold protein that is able to interact with several proteins, including PDE4B (Millar et al. 2005; Clapcote et al. 2007). The PDE4B-specific binding sites encompass point mutations in mouse DISC1 that confer phenotypes related to schizophrenia and depression (Murdoch et al. 2007). We speculate that functional variation in DISC1 and PDE4 will modulate their interaction and affect cAMP signaling.

PDE4D knockout mice exhibit significant changes in associative learning (i.e., fear conditioning) and a reduction of immobility in the forced-swim and tail suspension tasks (Rutten et al. 2008; Zhang et al. 2002a, b). Inhibition of the residual PDE4 subtypes in PDE4D knockout mice (i.e., PDE4A and PDE4B) by repeated treatment with rolipram does not result in further antidepressant-like effects on behavior (Zhang et al. 2002a, b). Consistent with this, PDE4D is highly expressed in the hippocampus, a sensitive structure in controlling the progression of depression and memory operations, including the formation of stable declarative (or explicit) memory in humans and spatial (or relational/contextual) memory in rodents. These results provide the evidence that PDE4D may play a crucial role in the mediation of antidepressant activity and memory (Rutten et al. 2008; Zhang et al. 2008). This is supported by findings that PDE4D is the most consistent subtype upregulated by repeated antidepressant treatment of mice, suggesting the involvement of this subtype in signaling pathways critical for these functions (Dlaboga et al. 2006).

It is possible that PDE4A also might be a particularly attractive target for regulating depression and associated cognitive deficits, since it is found to distribute in a similar pattern as PDE4D in the brain (Takahashi et al. 1999). Consistent with this notion, it has been found that the PDE4A subtype in neuronal cultures is regulated in response to changes in NMDA receptor function, suggesting a possible role in cAMP signaling in memory-related processes such as long-term potentiation (Hajjhussein et al. 2007). Evaluation of the behavioral phenotype of PDE4A knockout mice would begin to define the functional role for this subtype in the brain. Recent studies suggested that the PDE4A5 protein is significantly increased in memory deficits induced by sleep deprivation, which can be reversed by rolipram (Vecsey et al. 2009). The interaction between cognitive impairment and PDE4A5 shows that it may be possible to develop compounds that block either the activity or targeting of the PDE4A5 isoform for treatment of cognitive impairment and related sleep disorders.

3.5 PDE5

PDE5 was initially identified and characterized from platelets and then lung (Coquil et al. 1980; Francis et al. 1980). However, its regulation of smooth muscle

vasodilation is its key therapeutic action. The PDE5 selective inhibitors sildenafil, vardenafil, and tadalafil are used to manage erectile dysfunction and pulmonary hypertension. There are three variants of PDE5, PDE5A1, A2, and A3 that are distinguished based on their N-terminal sequence. PDE5A1 and PDE5A2 are ubiquitous, but PDE5A3 is specifically expressed in smooth muscles (Lin 2004). A single PDE5A gene encodes the three isoforms and the initial three exons (A1, A3, A2) encode the isoform-specific sequences.

Cyclic GMP levels are determined by the balance between activities of guanylyl cyclases and the PDEs that break down cGMP in the whole cell (Francis et al. 2010). The most common mechanism of modulation of PDE5 activity seems to be through N-terminal GAF domains that interact with cGMP and regulate catalytic activity (Heikaus et al. 2009). In mammals, PDE5 is found to contain N-terminal GAF A and GAF B domains (Bender and Beavo 2006; Martinez et al. 2002). Due to its significant expression in cerebellar Purkinje cells in the brain, PDE5 contributes to cognitive processes through cGMP regulation (Rapoport et al. 2000; Hartell 1996).

PDE5 inhibitors such as sildenafil and vardenafil have been shown to be not only effective in treatment of erectile dysfunction (Crowe and Streetman 2002; Fink et al. 2002), but also are candidate drugs for cognition enhancement (Prickaerts et al. 2004). PDE5 inhibitors are reported to improve memory performance, although it must be mentioned that an improved performance has been observed in learning tasks in the passive avoidance test but not in the water maze task (Prickaerts et al. 2004). Earlier studies suggested that vardenafil improves early processes of memory consolidation when it is administered immediately after the first trial in the novel object recognition task (Prickaerts et al. 2002). However, memory enhancing effects are not observed when vardenafil is administered at a later time point after training (3 or 6 h after the first trial) (Prickaerts et al. 2004; Rutten et al. 2007). These findings are in agreement with studies that suggest that vardenafil is able to attenuate the acute tryptophan depletion-induced object recognition impairment (van Donkelaar et al. 2008).

Sildenafil has been shown to ameliorate the deficit in performance in a T-maze task induced by a muscarinic acetylcholine receptor antagonist (Devan et al. 2004). It is also found to influence long-term memory retention in mice by modulating mechanisms involved in memory storage (Baratti and Boccia 1999). Other studies have shown that sildenafil improves acquisition and retention of memory in mice tested in the modified elevated plus maze (Singh and Parle 2003). Sildenafil administered immediately after training on the first day produces a dose-dependent improvement of memory during testing on the second day (Singh and Parle 2003). A recent study showed that sildenafil rescues synaptic and memory deficits in an APP/PS1 transgenic mouse model of amyloid deposition. This effect is associated with a long-lasting reduction of beta-amyloid levels after sildenafil treatment (Puzzo et al. 2009). Moreover, sildenafil can cross the blood–brain barrier and increase neurogenesis, which could have effects on memory (Uthayathas et al. 2007; Zhang et al. 2002a, b).

PDE5 inhibitors have also been proposed for the treatment of depression. Liebenberg and coworkers showed that sildenafil and tadalafil have significant

antidepressant-like effects in some animal models of depression (Liebenberg et al. 2010). However, further studies on the potential antidepressant-like effect of PDE5 inhibitors are needed.

3.6 PDE6

PDE6 expression is confined to the retina and pineal in some species and its inhibition is not directly relevant to psychopharmacology.

3.7 PDE7

The PDE7 gene family consists of two genes, PDE7A and PDE7B, with high affinity for cAMP, determined by its targeting domain at the N-terminal. Three splice variants of PDE7A are identified in humans, PDE7A1, PDE7A2, and PDE7A3, which differ in their N- and C-terminal regions (Han et al. 1997); the PDE7A1 promoter contains a cAMP-responsive element (Torrás-Llort and Azorin 2003). Besides its wide expression in the CNS, PDE7 mRNA and protein are also expressed in immune and proinflammatory cells. Some investigators have suggested that PDE7A may play a role in both activation and proliferation of T cells and in the regulation of human B-lymphocytes (Li et al. 1999; Lee et al. 2002; Kadoshima-Yamaoka et al. 2009), although some studies have questioned the importance of this enzyme in T-cell function (Smith et al. 2004; Yang et al. 2003; Guo et al. 2009). Altered immune and CNS interactions have been implicated in depression (Mendelovic et al. 1997, 1999), suggesting that PDE7 inhibition may affect mood by multiple mechanisms.

The highest levels of PDE7A mRNA expression are found in the hippocampus and olfactory bulb (Miro et al. 2001). Some overlap of the expression pattern can be observed when PDE7A mRNA localization is compared with that of the other cAMP-specific PDEs such as the PDE4 family (Miro et al. 2001; Pérez-Torres et al. 2001). The consistent colocalization with PDE4 mRNAs supports the hypothesis that PDE7A could have effects on memory impairment and depression. Previous studies demonstrated the cAMP/PKA/CREB pathway-mediated transcriptional activation of PDE7B is through the dopamine D1 receptor in primary striatal cultures (Reyes-Irisarri et al. 2005). Sasaki et al. (2004) examined the regional distribution and cellular localization of PDE7B mRNA in rat brain by *in situ* hybridization histochemistry. They found that 74 and 79% of the cells expressing PDE7B mRNA in striatum and olfactory tubercle are GABAergic cells (expressing glutamic acid decarboxylase mRNA). Although the role of PDE7B *in vivo* remains unclear, these results offer a neuroanatomical and neurochemical basis that supports the search for specific functions for the development of specific PDE7 inhibitors (Reyes-Irisarri et al. 2005).

3.8 *PDE8*

Although PDE8 is expressed in brain (Kobayashi et al. 2003), there are no published reports of the psychopharmacological effects of PDE8 inhibitors. Altered PDE8 mRNA expression in the postmortem hippocampus of patients with Alzheimer's disease (Pérez-Torres et al. 2003) suggests a potential role in cognitive function.

3.9 *PDE9*

BAY 73-6691 is a novel, potent, and selective PDE9 inhibitor that is currently under preclinical development for the treatment of Alzheimer's disease (Wunder et al. 2005). BAY 73-6691 enhances acquisition, consolidation, and retention of long-term memory in a social recognition task and tends to enhance long-term memory in an object recognition task (van der Staay et al. 2008). It attenuates the scopolamine-induced retention deficit in a passive avoidance task, and MK-801-induced short-term memory deficit in a T-maze alternation task. A recent study reported that an inhibitor of PDE9 improves learning and memory in older rats but not in young ones (Domek-Łopacińska and Strosznajder 2010). An increase in PDE9 expression and activity and a decrease in cGMP concentration are also found in the aged rat brain in this study. The results support the notion that PDE9 inhibition may be a novel means for treating memory deficits that are associated with aging and neurodegenerative disorders such as Alzheimer's disease.

3.10 *PDE10*

The PDE10 family was originally identified simultaneously by three different groups (Fujishige et al. 1999; Loughney et al. 1999; Soderling et al. 1999). There is only one gene in the PDE10 family, PDE10A, which hydrolyzes both cAMP and cGMP *in vitro*. The four major isoforms of PDE10A are PDE10A1–4, which differ at their N- and C-termini, whereas the GAF domains are identical. The GAF domain of PDE10 binds and regulates via cAMP rather than cGMP (Gross-Langenhoff et al. 2006). This occurs for a chimeric protein of the PDE10 GAF domains with a bacterial adenylyl cyclase catalytic domain as the binding of cAMP-stimulated cyclase catalytic activity (Bender and Beavo 2006). Whether there is a functional allosteric effect of the cAMP-stimulated GAF domain for the regulation the intracellular PDE10 remains unknown.

In rodents, PDE10A mRNA is highly expressed in the caudate-putamen, hippocampus, cerebral cortex, olfactory tubercle, thalamus, and spinal cord (Hebb et al. 2004; Hu et al. 2004; O'Conner et al. 2004; Siuciak et al. 2006; Xie et al. 2006).

PDE10A protein is observed in γ -aminobutyric acid-(GABA) containing medium spiny projection neurons of the mammalian striatum, which is the core site for information integration and modulating motor and cognitive processing (Seeger et al. 2003; Menniti et al. 2006). Therefore, it seems that PDE10A is an important regulator of cGMP in the striatum and may be involved in the physiological regulation of motor and cognitive function (Lehericy and Gerardin 2002).

It is reported that inhibition of PDE10A causes a reduction of locomotor activity and a failure to respond to psychomotor stimulants. Chronic PDE10A inhibition produces a variety of behavioral and central neurochemical effects, which are exacerbated by stress (Hebb et al. 2008). PDE10A knockout mice exhibit decreased exploratory activity and a significant delay in the acquisition of conditioned avoidance behavior when compared to wild-type mice (Siuciak et al. 2006). PDE10A inhibition in primary neuronal cultures increases phosphorylation of CREB in the nucleus; however, the functional significance of this effect is unclear (Menniti et al. 2006). The most consistent series of findings regarding PDE10 inhibitors suggests their potential for treatment of symptoms of schizophrenia.

3.11 PDE11

The most recently discovered of the PDE families is PDE11A, which catalyzes the hydrolysis of cAMP and cGMP equally well (Francis 2005; Yuasa et al. 2001; Weeks et al. 2007). The first report of PDE11A was by Fawcett et al. (2000), who identified a partial sequence from a commercially available expressed sequence tag database based on homology with other mammalian PDEs (Makhlouf et al. 2006). Four splicing variants of PDE11A have been discovered, PDE11A1–4. PDE11A4 is the longest variant with two N-terminal GAF domains, while the other variants are truncated into shorter lengths (Bender and Beavo 2006).

Initial studies suggested that PDE11A is found in skeletal muscle, prostate, testis, pancreas, and salivary glands. Subsequent work showed that PDE11A is also found in the brain, pituitary, and spinal cord (Fawcett et al. 2000; Yuasa et al. 2001). Recent findings reveal that PDE11A mRNA and protein in the brain are largely restricted to hippocampus and subiculum, suggesting a potential role in mood and cognitive function (Kelly et al. 2010). This group showed that PDE11A knockout mice exhibit subtle behavioral changes relevant to psychiatric disease, including hyperactivity in an open field, increased sensitivity to the glutamate NMDA receptor antagonist MK-801, and deficits in social behaviors (social odor recognition memory and social avoidance). It has been speculated that PDE11A may be one of the several genes playing a role in the multifactorial origin of psychiatric diseases such as schizophrenia. A single nucleotide polymorphism (SNP) analysis also suggests that PDE11 may be involved in processes affecting depression and antidepressant drug response (Wong et al. 2006).

Table 2 shows an overview of the effects of PDE inhibitors on behaviors related to mood and cognitive disorders.

Table 2 Overview of the effects of PDE inhibitors on mood and related cognitive disorders

PDE inhibitors	Drug	Model	Task	Results	References
PDE1	Vinpocetine	Streptozotocin impaired rat	Morris water maze test	Vinpocetine improves memory consolidation	Deshmukh et al. (2009)
PDE2	BAY60-7550	MK-801 impaired mouse	Object recognition task; T-maze test	BAY60-7550 reverses MK801-induced memory deficits	Boess et al. (2004)
		Age-impaired rat	Object recognition task	BAY60-7550 improves acquisition and consolidation	Domek-Lopacińska and Strosznajder (2008)
		Oxidative stress impaired rat	Elevated plus maze; open-field test	BAY60-7550 reverses the oxidative stress induced anxiety-like behaviors in mice	Masood et al. (2008)
	ND7001	Oxidative stress impaired rat	Elevated plus maze; open-field test	ND7001 reverses the oxidative stress induced anxiety-like behaviors in mice	Masood et al. (2008)
PDE4	Rolipram	Scopolamine impaired rat	Radial maze test	Rolipram reverses Scopolamine-induced deficits in both working memory and reference memory	Zhang and O'Donnell (2000)
		MK-801 impaired rat	Three-panel runway task; radial-arm maze test; passive avoidance test	Rolipram reverses the working memory deficits caused by MK-801	Zhang et al. (2000, 2005), Reneerkens et al. (2009)
		Young rhesus monkey	T-maze test	Rolipram improves working memory in young rhesus monkeys but not in aged ones	Ramos et al. (2003)
	MEM1018	Beta-amyloid impaired rat	Morris water maze test; passive avoidance test	Rolipram reverses memory deficits induced by Abeta 25-35	Cheng et al. 2010
		MK-801 impaired rat	Radial-arm maze test; step through inhibitory avoidance task	MEM1018 improves the memory processes	Zhang et al. (2005)
	MEM1091	MK-801 impaired rat	Radial-arm maze test; step through inhibitory avoidance task	MEM1091 improves the memory process	Zhang et al. 2005

(continued)

Table 2 (continued)

PDE inhibitors	Drug	Model	Task	Results	References
	PDE4B knockout mouse		Hole board test; light-dark transition test; open-field test	PDE4B knockout mouse decreases head-dips and time spent in head-dipping in the hole board test, reduces transitions and time on the light side in the light-dark transition test, and decreases initial exploration and rears in the open-field test	Zhang et al. (2008)
	PDE4D knockout mouse		Forced swim task; tail suspension task	PDE4D knockout mouse exhibits significant changes in associative learning (i.e. fear conditioning) and a reduction of immobility in the tail suspension and forced swim tasks	Ruitten et al. (2008), Zhang et al. (2002a, b)
PDE5	Sildenafil (Viagra)	Muscarinic acetylcholine receptor antagonist impaired rat mouse	T-maze task	Sildenafil ameliorates memory deficits induced by a muscarinic acetylcholine receptor antagonist	Devan et al. (2004)
		APP/PS1 transgenic mouse	Fear conditioning task; Morris water maze test	Sildenafil rescues synaptic and memory (fear memory and working memory) deficits in an APP/PS1 transgenic mouse model of amyloid deposition	Puzzo et al. (2009)
	Vardenafil	Tryptophan deleted rat	Modified elevated plus maze	Sildenafil improves acquisition and retention of memory in mice	Singh and Parle (2003)
			Object recognition task	Vardenafil attenuates the acute tryptophan depletion induced object recognition impairment	van Donkelaar et al. (2008)

PDE9	BAY 73-6691	Scopolamine impaired rat; MK-801 impaired rat	Passive avoidance test; T-maze test; object recognition task; social recognition task	BAY 73-6691 enhances acquisition, consolidation, and retention of long-term memory in a social recognition task and tends to enhance long-term memory in an object recognition task	van der Staay et al. (2008)
PDE10		PDE10A knockout mouse	Passive avoidance test	PDE10A knockout mouse exhibits decreased exploratory activity and a significant delay in the acquisition of conditioned avoidance behavior when compared to wild-type mice	Siuciak et al. (2006)
PDE11		PDE11A knockout mouse	Open-field test	PDE11A knockout mice show subtle psychiatric disease-related behaviors, including hyperactivity in an open field, increases sensitivity to the glutamate NMDA receptor antagonist MK-801, as well as deficits in social behaviors (social odor recognition memory and social avoidance)	Kelly et al. (2010)

4 Regulation of Signaling Pathways Involved in Mood and Cognition by PDE Inhibitors

Cyclic AMP and cGMP, as two critical second messengers, play an important role in regulating CNS functions, including emotion-related learning and memory, and nerve growth regeneration (Houslay et al. 2005; Tanis and Duman 2007; Teng and Tang 2006). The changes in cAMP and cGMP levels are caused by both accumulation resulting from the synthetic cyclases and degradation, which is regulated by PDEs (Conti et al. 1995). Actually, the manipulation of cAMP/cGMP degradation and creation of different local cyclic nucleotide concentrations for selective actions seem to play a more important role, which makes PDEs promising drug targets (Bender and Beavo 2006). There is also ample evidence that cyclic nucleotides are involved in LTP, since enhanced cAMP/cGMP concentrations can improve LTP and increase synaptic plasticity; this suggests a potential mechanism for the use of PDE inhibitors to treat memory dysfunctions (Impey et al. 1996; Frey et al. 1993; Monfort et al. 2004; Bon and Garthwaite 2003). Since PDEs are widely distributed in brain and are key enzymes in the complex cAMP/cGMP regulatory systems that transduce membrane signals to the nucleus to regulate gene expression, they have the potential to effect long-term changes in neuronal function. Therefore, PDEs have become interesting targets in psychiatric and neurological disorders (Esposito et al. 2009); the exception to this is PDE6, which is localized to the retina (Taylor et al. 2001). In addition, it has been suggested that drugs that target second messengers might have higher efficacy since they can modulate multiple biochemical pathways (Blokland et al. 2006).

4.1 PDE Inhibitors and the cAMP-PKA Pathway

Cyclic AMP, through activating cAMP-dependent protein kinase, can stimulate the transcription factor CREB, which regulates the transcription of many genes, such as brain-derived neurotrophic factor (BDNF); this cascade has been suggested to be involved in the pathophysiology of mood and cognitive disorders (Lonze and Ginty 2002). Evidence is accumulating that PDE inhibitors modulate the activity of cAMP/cGMP-mediated signaling pathways and thus regulate CREB phosphorylation and the downstream effectors (Reneerkens et al. 2009). The phosphorylation of CREB at Ser-133 is required for CREB activation that has been implicated in plasticity of synaptic function that is important in memory and cognition (Lonze and Ginty 2002). Dominant-negative CREB mutation or key CREB isoforms deletions can affect memory formation and long-term synaptic changes in mice (Chen et al. 2003). Moreover, CREB affects the transcription of many critical genes, such as BDNF, which is a major neurotrophin in the brain and has been shown to be involved in etiology of depression and actions of antidepressants (Duman et al. 1999; Egan et al. 2003; Sklar et al. 2002). Decreased phosphorylated

CREB levels may contribute to memory deficits, mood disorders, and altered nerve growth and regeneration (Monti et al. 2006).

PDE4, which hydrolyzes cAMP specifically, regulates many crucial signaling cascades involved in learning and memory (Ahmed and Frey 2005; Blokland et al. 2006; Menniti et al. 2006). PDE4 inhibitors, by increasing the cAMP concentration and activating CREB phosphorylation, enhance LTP and improve spatial and associative memory in mice (Barad et al. 1998; Bach et al. 1999). Mice carrying a truncated form of CREB protein-binding protein (CBP) show several developmental abnormalities similar to patients with mental retardation. CBP is a 265 kDa nuclear protein that is a coactivator molecule for a number of transcription factors, including CREB and several nuclear receptors. Memory deficits in step through passive avoidance test and fear conditioning test are found in mice with mutations of CBP (Oike et al. 1999); rolipram ameliorates impairments of long-term memory and CREB signaling caused by CBP mutation (Bourtchouladze et al. 2003).

PDE4 is also implicated in hippocampal neurogenesis, which is associated with memory and mood disorders (Epp et al. 2007). Repeated administration of rolipram improves the survival ratio of newborn neurons through activating cAMP/CREB signaling, as evidenced by increased cAMP, PKA activity, and pCREB expression (Fujioka et al. 2004; Li et al. 2009). Interestingly, it was found that the effect of rolipram on CREB is in a brain region-specific manner, in which increased pCREB expression is observed in the hippocampus but not the frontal cortex. However, it is reported that the pCREB expression in newborn neurons in the hippocampus only partially contributes to the behavioral effects of rolipram (Li et al. 2009). When coadministered with the tricyclic antidepressant imipramine, rolipram significantly increases in the level of pCREB and its downstream effector BDNF in the frontal cortex and hippocampus of learned helplessness rats. Moreover, the escape failures in these rats are completely eliminated by coadministration of rolipram with imipramine (Itoh et al. 2004). These results indicate that activation of CREB may be involved in the antidepressant effects involving PDE4 and cAMP signaling.

Recent studies show that PDE7 and PDE8, which are cAMP-specific enzymes, are thought to regulate the cAMP levels in the human striatum and dentate gyrus (Sasaki et al. 2004). Enhanced levels of PDE7 and PDE8 mRNAs are observed in the hippocampus of patients with Alzheimer's disease and dementia (Pérez-Torres et al. 2003). Therefore, selective PDE7 and PDE8 inhibitors might be able to enhance cAMP concentrations without inducing the side effects associated with PDE4 inhibitors (Sasaki et al. 2000).

4.2 PDE Inhibitors and the MAPK-ERK Pathway

The activation of CREB also occurs via the MAPK/ERK pathway; MAPK inhibitors reduce CREB phosphorylation when given together with PKA inhibitors (Gao et al. 2004). The MAPK pathway is thought to stimulate CREB via activation of p90 ribosomal S6 kinase 2 (RSK2) (Anjum and Blenis 2008). It has been

reported that NMDA receptor activation, which activates MAPK, is required for LTP induction (Miyamoto 2006). Moreover, the specific NMDA receptor antagonist AP5 inhibits the effect of rolipram on LTP (Navakkode et al. 2005). This result is consistent with the report that rolipram reverses the memory deficits induced by the NMDA receptor antagonist MK-801 (Zhang et al. 2000, 2005), suggesting that PDE4 inhibitors might trigger the NMDA receptor-mediated MAPK/ERK cascade, and thus phosphorylate CREB (Valera et al. 2008). However, depending on which isoforms of Raf are present and to what pools of ERK they interact with (Schaak et al. 2000), cAMP can also activate ERK through stimulation of B-Raf (Obara et al. 2009), while PKA-mediated phosphorylation of C-Raf inhibits its functioning and thus ERK activation (Dumaz and Marais 2003). Indeed, PDE4 inhibitors have been shown to inhibit the MAPK signaling pathway, which is probably due to PKA-mediated inhibition of Raf-1 activity (Matousovic et al. 1995). Therefore, PDEs inhibitors might regulate MAPK-ERK-CREB signaling via cAMP and indirectly by altering NMDA receptor function.

Additionally, there is also crosstalk between the ERK and cAMP pathways as ERK can phosphorylate a conserved serine within the PDE4 catalytic site, whereby it activates short forms and inhibits long isoforms, providing either positive or negative interactions dependent upon the target PDE4 isoform (Baillie et al. 2000; Mackenzie et al. 2000).

4.3 PDE Inhibitors and the NO/cGMP/PKG Pathway

Nitric oxide plays an important role in LTP; inhibition of nitric acid synthase (NOS) reduces LTP significantly (Doyle et al. 1996; Haley et al. 1993). cGMP is a downstream effector of nitric oxide (NO); stimulation of NMDA receptors by glutamate results in an increase in intracellular calcium, which activates NOS and leads to enhanced production of NO and activation of soluble guanylyl cyclase (sGC), which catalyzes cGMP synthesis (Monfort et al. 2004).

There is substantial evidence showing important functions of cGMP-specific PDEs in memory and synaptic plasticity (Blokland et al. 2006; Menniti et al. 2007). Inhibition of the breakdown of cGMP by PDE5 inhibitors facilitates memory consolidation in the rodent novel object recognition task (Prickaerts et al. 2002, 2004). It has been demonstrated that the PDE5 inhibitor zaprinast increases the NO-activated cGMP response in the hippocampus (De Vente et al. 1996; Van Staveren et al. 2001), which suggests that the memory-enhancing effect of PDE5 inhibitors might be mediated through NO-cGMP signaling. Prickaerts and coworkers (2002, 2004) have shown that cGMP/PKG pathway is mainly involved in presynaptic changes and in the early phase of the signaling pathway activated by PDE5 inhibitors.

It is known that neurogenesis plays a role in synaptic plasticity, memory, and mood disorders (Bruehl-Jungerman et al. 2006, 2007). Some studies also show that treatment of mood disorders such as depression is related to neurogenesis and synaptic plasticity (Paizanis et al. 2007). Zhang et al. showed that PDE5 inhibitors

sildenafil and tadalafil stimulate neurogenesis after stroke in rats (Zhang et al. 2002a, b, 2006). In addition, PDE5 inhibition has been reported to modulate the antidepressant effect of venlafaxine and bupropion (Dhir and Kulkarni 2007a, b). Brink and coworkers (2008) only observed an antidepressant-like effect of sildenafil after blocking central muscarinic receptors, indicating the crosstalk with cholinergic pathways.

PDE2 might be a link between cAMP and cGMP signaling mechanisms in neurons (Hajjhussein et al. 2007). Studies have shown that PDE2 inhibitor BAY 60-7550, which exerts positive effects on learning and memory, enhances the induction of LTP in hippocampus (Boess et al. 2004). PDE2 inhibitors also increase NOS activity (Domek-Łopacińska and Strosznajder 2008), suggesting a possible feedback effect on NO-mediated cGMP signaling. In addition, Bay 60-7550 also prevents neurochemical and anxiogenic-like behavioral effects induced by oxidative stress in mice; this appears to be mediated through NO-cGMP signaling (Masood et al. 2008). Recent research further demonstrates a key role for cGMP signaling in the effects of PDE2 inhibitors on neurons as well as on their anxiolytic effects on behavior (Masood et al. 2009). Thus, cGMP-specific PDEs might serve as potential therapeutic targets to improve neuronal function in neurological and psychiatric disorders, particularly those where increased oxidative stress is a contributing factor.

Elevated cGMP levels are thought to act in part through activating cGMP-dependent protein kinases (PKGs), which act in parallel with PKA to phosphorylate the transcription factor CREB (Pilz and Broderick 2005; Lu et al. 1999). Previous studies have shown that cGMP/PKG/CREB pathway, just like cAMP/PKA/CREB cascade, can induce the synthesis of proteins which are important for LTP and memory consolidation (Abel et al. 1997). Since beta-amyloid protein has been demonstrated to reduce CREB phosphorylation (Puzzo et al. 2005), enhancers of cAMP/PKA/CREB or cGMP/PKG/CREB may represent new classes of anti-Alzheimer's disease drugs by targeting the downstream consequences of beta-amyloid (Rutten et al. 2007). Consistent with this, PDE5 inhibitors, which are thought to activate cGMP/PKG/CREB, have been proposed to be memory enhancers (Rose et al. 2005). Compared to PDE4 inhibitors, which induce various side effects, PDE5 inhibitors might have broader therapeutic application in cognition and mood disorders (Souness et al. 2000). PDE10 is a dual-substrate enzyme, and PDE10A has been shown to hydrolyze both cAMP and cGMP in the medium spiny neurons (Strick et al. 2010). PDE10A inhibition in primary cortical and hippocampal neurons increases CREB phosphorylation in the nucleus (Menniti et al. 2006). Although the mechanism underlying this process is still unclear, PDE10A is becoming an interesting target for understanding and treating CNS disorders.

4.4 PDE Inhibitors and Neuroplasticity

Apoptosis or programmed cell death is essential for the normal functioning and development of the nervous system (Quiroz et al. 2010). Neuronal apoptosis is

also a prominent feature in a variety of neurodegenerative and neuropsychiatric disorders (Viana et al. 2010) with various studies revealing the role of apoptosis in the pathology of depression and anxiety where cGMP is likely to be inhibited (Lucassen 2004; Einat et al. 2005). It is possible that PDE activities in these neuropsychiatric disorders may be altered leading to stimulation of a proapoptotic cascade and symptoms associated with impaired learning and memory, depression, or anxiety.

Neuronal plasticity or remodeling is a critical process that underlies normal CNS function by which the brain acquires information and makes the appropriate adaptive responses in future-related settings (Reinés et al. 2008). Dysfunction of neuronal plasticity could therefore contribute to the pathophysiology of mood disorders and recovery could occur by induction of the appropriate plasticity or remodeling phenomena (Akhondzadeh 1999; Duman 2002). Two aspects of neuronal plasticity are important for information processing: plasticity of intrinsic excitability, i.e., the change in ion channel properties; and synaptic plasticity, i.e., the change in the strength of synapses between neurons (Kotaleski and Blackwell Kim 2010). Synaptic plasticity is thought to underlie learning and memory, but the complexity of the interactions between ion channels, enzymes, and genes that are involved in synaptic plasticity impedes a deep understanding of this phenomenon (Pereira and Furlan 2010). Recently, Chen and colleagues (2010) found that inhibition of mossy fiber LTP by acute stress originates from a sustained increase in PDE4 activity, which accelerates the metabolism of cAMP to adenosine, in turn triggering an adenosine receptor-mediated impairment of transmitter release machinery. These results are supported by a more recent study, which indicated that, PDE, cAMP, and its main effector PKA are critical for several aspects of neuronal function including synaptic plasticity (Oliveira et al. 2010).

5 Future Directions

Over the last five decades, great progress has been made in understanding the roles of PDEs and their inhibitors in regulating CNS functions that are important for the pathophysiology and treatment of neurological and psychiatric disorders. However, the understanding of PDE function in the CNS is incomplete and requires further investigation. First, it is necessary to obtain more knowledge about the localization of specific PDE isoforms in different brain regions, cell types, and intracellular compartments. Second, it would be helpful for more powerful tools to be developed to characterize and manipulate individual PDE isoforms *in situ* and to identify their involvement in signaling machinery. Third, it will be important that novel and selective inhibitors be developed that have potential to selectively affect individual subtypes and isoforms or to alter PDE function through allosteric interactions or by disrupting interactions with scaffolding or auxiliary proteins. Such information and tools will provide a better basis for the development of drugs to treat neurological and psychiatric disorders.

References

- Abel T, Nguyen PV, Barad M, Deuel TAS, Kandel ER (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus based long term memory. *Cell* 88:615–626
- Ahmed T, Frey JU (2005) Phosphodiesterase 4B (PDE4B) and cAMP-level regulation within different tissue fractions of rat hippocampal slices during long-term potentiation in vitro. *Brain Res* 1041:212–222
- Aizawa T, Wei H, Miano JM, Abe J, Berk BC, Yan C (2003) Role of phosphodiesterase 3 in NO/cGMP-mediated antiinflammatory effects in vascular smooth muscle cells. *Circ Res* 93:406–413
- Akhondzadeh S (1999) Hippocampal synaptic plasticity and cognition. *J Clin Pharm Ther* 24:241–248
- Anjum R, Blenis J (2008) The RSK family of kinases: emerging roles in cellular signaling. *Nat Rev Mol Cell Biol* 9:747–758
- Bach ME, Bach ME, Barad M, Son H, Zhuo M, Lu YF, Shih R, Mansuy I, Hawkins RD, Kandel ER (1999) Age-related defects in spatial memory are correlated with defects in the late phase of hippocampal long-term potentiation in vitro and are attenuated by drugs that enhance the cAMP signaling pathway. *Proc Natl Acad Sci USA* 96:5280–5285
- Baillie GS, MacKenzie SJ, McPhee I, Houslay MD (2000) Sub-family selective actions in the ability of Erk2 MAP kinase to phosphorylate and regulate the activity of PDE4 cyclic AMP-specific phosphodiesterases. *Br J Pharmacol* 13:811–819
- Barad M, Bourchouladze R, Winder DG, Golan H, Kandel E (1998) Rolipram, a type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory. *Proc Natl Acad Sci USA* 95:15020–15025
- Baratti CM, Boccia MM (1999) Effects of sildenafil 1 on long-term retention of an inhibitory avoidance response in mice. *Behav Pharmacol* 10:731–737
- Barnette MS, Underwood DC (2000) New phosphodiesterase inhibitors as therapeutics for the treatment of chronic lung disease. *Curr Opin Pulm Med* 6:164–169
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Birnbaum SG, Gobseske KT, Auerbach J, Taylor JR, Arnsten AFT (1999) A role for norepinephrine in stress-induced cognitive deficits: alpha-1-adrenoceptor mediation in prefrontal cortex. *Biol Psychiatry* 46:1266–1274
- Blokland A, Schreiber R, Prickaerts J (2006) Improving memory: a role for phosphodiesterases. *Curr Pharm Des* 12:2511–2523
- Boess FG, Hendrix M, van der Staay FJ, Erb C, Schreiber R, van Staveren W, de Vente J, Prickaerts J, Blokland A, Koenig G (2004) Inhibition of phosphodiesterase 2 increases neuronal cGMP, synaptic plasticity and memory performance. *Neuropharmacology* 47:1081–1092
- Bolger G, Michaeli T, Martins T, St John T, Steiner B, Rodgers L, Riggs M, Wigler M, Ferguson K (1993) A family of human phosphodiesterases homologous to the dunce learning and memory gene product of *Drosophila melanogaster* are potential targets for antidepressant drugs. *Mol Cell Biol* 13:6558–6571
- Bolger GB, Rodgers L, Riggs M (1994) Differential CNS expression of alternative mRNA isoforms of the mammalian genes encoding cAMP-specific phosphodiesterases. *Gene* 149:237–244
- Bon CL, Garthwaite J (2003) On the role of nitric oxide in hippocampal long-term potentiation. *J Neurosci* 23:1941–1948
- Bos JL (2006) Epac proteins: multi-purpose cAMP targets. *Trends Biochem Sci* 31:680–686
- Bourchouladze R, Lidge R, Catapano R, Stanley J, Gossweiler S, Romashko D, Scott R (2003) A mouse model of Rubinstein–Taybi syndrome: defective long-term memory is ameliorated by inhibitors of phosphodiesterase 4. *Proc Natl Acad Sci USA* 100:10518–10522

- Bouton ME, Westbrook RF, Corcoran KA, Maren S (2006) Contextual and temporal modulation of extinction: behavioral and biological mechanisms. *Biol Psychiatry* 60:352–360
- Brink CB, Clapton JD, Eagar BE, Harvey BH (2008) Appearance of antidepressant-like effect by sildenafil in rats after central muscarinic receptor blockade: evidence from behavioural and neuro-receptor studies. *J Neural Transm* 115:117–125
- Bruel-Jungerman E, Davis S, Rampon C, Laroche S (2006) Long-term potentiation enhances neurogenesis in the adult dentate gyrus. *J Neurosci* 26:5888–5893
- Bruel-Jungerman E, Rampon C, Laroche S (2007) Adult hippocampal neurogenesis, synaptic plasticity and memory: facts and hypotheses. *Rev Neurosci* 18:93–114
- Bunge SA, Ochsner KN, Desmond JE, Glover GH, Gabrieli JD (2001) Prefrontal regions involved in keeping information in and out of mind. *Brain* 124:2074–2086
- Cassel JC, Cassel S, Galani R, Kelche C, Will B, Jarrard L (1998) Fimbria-fornix vs. selective hippocampal lesions in rats: effects on locomotor activity and spatial learning and memory. *Neurobiol Learn Mem* 69:22–45
- Chen A, Muzzio IA, Malleret G, Bartsch D, Verbitsky M, Pavlidis P, Yonan AL, Vronskaya S, Grody MB, Cepeda I, Gilliam TC, Kandel ER (2003) Inducible enhancement of memory storage and synaptic plasticity in transgenic mice expressing an inhibitor of ATF4 (CREB-2) and C/EBP proteins. *Neuron* 39:655–669
- Chen CC, Yang CH, Huang CC, Hsu KS (2010) Acute stress impairs hippocampal mossy fiber-CA3 long-term potentiation by enhancing cAMP-specific phosphodiesterase 4 activity. *Neuropsychopharmacology* 35:1605–1617
- Cheng YF, Wang C, Lin HB, Li YF, Huang Y, Xu JP, Zhang HT (2010) Inhibition of phosphodiesterase-4 reverses memory deficits produced by Abeta 25-35 or Abeta1-40 peptide in rats. *Psychopharmacology* 212:181–191
- Cherry JA, Davis RL (1999) Cyclic AMP phosphodiesterases are located in region of the mouse brain associated with reinforcement, movement, and effect. *J Comp Neurol* 407: 287–301
- Clapcote SJ, Lipina TV, Millar JK, Mackie S, Christie S, Ogawa F, Lerch JP, Trimble K, Uchiyama M, Sakuraba Y, Kaneda H, Shiroishi T, Houslay MD, Henkelman RM, Sled JG, Gondo Y, Porteous DJ, Roder JC (2007) Behavioral phenotypes of *Disc1* missense mutations in mice. *Neuron* 54:387–402
- Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. *Annu Rev Biochem* 76:481–511
- Conti M, Nemoz G, Sette C, Vicini E (1995) Recent progress in understanding the hormonal regulation of phosphodiesterases. *Endocr Rev* 16:370–389
- Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C (2003) Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. *J Biol Chem* 278: 5493–5496
- Coquil JF, Franks DJ, Wells JN, Dupuis M, Hamet P (1980) Characteristics of a new binding protein distinct from the kinase for guanosine 3, 5-monophosphate in rat platelets. *Biochim Biophys Acta* 631:148–165
- Coven E, Ni Y, Widnell KL, Chen J, Walker WH, Habener JF, Nestler EJ (1998) Cell type-specific regulation of CREB gene expression: mutational analysis of CREB promoter activity. *J Neurochem* 71:1865–1874
- Crowe SM, Streetman DS (2002) Sildenafil for male erectile dysfunction: a systematic review and meta-analysis. *Arch Intern Med* 162:1349–1360
- De Vente J, Hopkins DA, Markerink-van Ittersum M, Steinbusch HWM (1996) Effects of the 3', 5'-phosphodiesterase inhibitors isobutylmethylxanthine and zaprinast on NO-mediated cGMP accumulation in the hippocampus slice preparation: an immunocytochemical study. *J Chem Neuroanat* 10:241–248
- DeNoble VJ (1987) Vinpocetine enhances retrieval of a step-through passive avoidance response in rats. *Pharmacol Biochem Behav* 26:183–186

- Deshmukh R, Sharma V, Mehan S, Sharma N, Bedi KL (2009) Amelioration of intracerebroventricular streptozotocin induced cognitive dysfunction and oxidative stress by vinpocetine – a PDE1 inhibitor. *Eur J Pharmacol* 620:49–56
- Devan BD, Sierra-Mercado D Jr, Jimenez M, Bowker JL, Duffy KB, Spangler EL, Ingram DK (2004) Phosphodiesterase inhibition by sildenafil citrate attenuates the learning impairment induced by blockade of cholinergic muscarinic receptors in rats. *Pharmacol Biochem Behav* 79:691–699
- Dhir A, Kulkarni SK (2007a) Effect of addition of yohimbine (alpha-2-receptor antagonist) to the antidepressant activity of fluoxetine or venlafaxine in the mouse forced swim test. *Pharmacology* 80:239–243
- Dhir A, Kulkarni SK (2007b) Involvement of nitric oxide (NO) signaling pathway in the antidepressant action of bupropion, a dopamine reuptake inhibitor. *Eur J Pharmacol* 568:177–185
- Dlaboga D, Hajjhussein H, O'Donnell JM (2006) Regulation of phosphodiesterase-4 (PDE4) expression in mouse brain by repeat antidepressant treatment: comparison with rolipram. *Brain Res* 1096:104–112
- Domek-Lopacińska KU, Strosznajder JB (2008) The effect of selective inhibition of cyclic GMP hydrolyzing phosphodiesterases 2 and 5 on learning and memory processes and nitric oxide synthase activity in brain during aging. *Brain Res* 1216:68–77
- Domek-Lopacińska KU, Strosznajder JB (2010) Cyclic GMP and nitric oxide synthase in aging and Alzheimer's disease. *Mol Neurobiol* 41:129–137
- Doyle C, Hölscher C, Rowan MJ, Anwyl R (1996) The selective neuronal NO synthase inhibitor 7-nitroindazole blocks both long-term potentiation and depotentiation of field EPSPs in rat hippocampal CA1 in vivo. *J Neurosci* 16:418–424
- Duman RS (2002) Pathophysiology of depression: the concept of synaptic plasticity. *Eur Psychiatry* 17:306–310
- Duman RS, Malberg J, Thome J (1999) Neural plasticity to stress and antidepressant treatment. *Biol Psychiatry* 46:1181–1191
- Dumaz N, Marais R (2003) Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras. *J Biol Chem* 278:29819–29823
- Dunkern TR, Hatzelmann A (2007) Characterization of inhibitors of phosphodiesterase 1C on a human cellular system. *FEBS J* 274:4812–4824
- Egan M, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M (2003) The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112:257–269
- Einat H, Yuan P, Manji HK (2005) Increased anxiety-like behaviors and mitochondrial dysfunction in mice with targeted mutation of the Bcl-2 gene: further support for the involvement of mitochondrial function in anxiety disorders. *Behav Brain Res* 165:172–180
- Engels P, Abdel'Al S, Hulley P, Lübbert H (1995) Brain distribution of four rat homologues of the *Drosophila dunce* cAMP phosphodiesterase. *J Neurosci Res* 41:169–178
- Epp JR, Spritzer MD, Galea LA (2007) Hippocampus-dependent learning promotes survival of new neurons in the dentate gyrus at a specific time during cell maturation. *Neuroscience* 149:273–285
- Esposito K, Reiersen GW, Luo HR, Wu GS, Licinio J, Wong ML (2009) Phosphodiesterase genes and antidepressant treatment response: a review. *Ann Med* 41:177–185
- Fatemi SH, King DP, Reutiman TJ, Folsom TD, Laurence JA, Lee S, Fan YT, Paciga SA, Conti M, Menniti FS (2008) PDE4B polymorphisms and decreased PDE4B expression are associated with schizophrenia. *Schizophr Res* 101:36–49
- Fawcett L, Baxendale R, Stacey P, McGrouther C, Harrow I, Soderling S, Hetman J, Beavo JA, Phillips SC (2000) Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proc Natl Acad Sci USA* 97:3702–3707

- Filgueiras CC, Krahe TE, Medina AE (2010) Phosphodiesterase type 1 inhibition improves learning in rats exposed to alcohol during the third trimester equivalent of human gestation. *Neurosci Lett* 473:202–207
- Fink HA, Mac Donald R, Rutks IR, Nelson DB, Wilt TJ (2002) Sildenafil for male erectile dysfunction: a systematic review and meta-analysis. *Arch Intern Med* 162:1349–1360
- Francis SH (2005) Phosphodiesterase 11 (PDE11): is it a player in human testicular function? *Int J Impot Res* 17:467–468
- Francis SH, Lincoln TM, Corbin JD (1980) Characterization of a novel cGMP binding protein from rat lung. *J Biol Chem* 255:620–626
- Francis SH, Busch JL, Corbin JD (2010) cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev* 62:525–563
- Frey U, Huang Y, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science* 260:1661–1664
- Fujioka T, Fujioka A, Duman RS (2004) Activation of cAMP signaling facilitates the morphological maturation of newborn neurons in adult hippocampus. *J Neurosci* 24:319–328
- Fujishige K, Kotera J, Omori K (1999) Striatum- and testis-specific phosphodiesterase PDE10A isolation and characterization of a rat PDE 10A. *Eur J Biochem* 266:1118–1127
- Gao Y, Deng K, Hou J, Bryson JB, Barco A, Nikulina E, Spencer T, Mellado W, Kandel ER, Filbin MT (2004) Activated CREB is sufficient to overcome inhibitors in myelin and promote spinal axon regeneration in vivo. *Neuron* 44:609–621
- Garthwaite J, Boulton CL (1995) Nitric oxide signaling in the central nervous system. *Annu Rev Physiol* 57:683–706
- Gong B, Vitolo OV, Trinchese F, Liu S, Shelanski M, Arancio O (2004) Persistent improvement in synaptic and cognitive functions in an Alzheimer mouse model after rolipram treatment. *J Clin Invest* 114:1624–1634
- Gross-Langenhoff M, Hofbauer K, Weber J, Schultz A, Schultz JE (2006) camp is a ligand for the tandem GAF domain of human phosphodiesterase 10 and cGMP for the tandem GAF domain of phosphodiesterase 11. *J Biol Chem* 281:2841–2846
- Guo J, Watson A, Kempson J, Carlsen M, Barbosa J, Stebbins K, Lee D, Dodd J, Nadler SG, McKinnon M, Barrish J, Pitts WJ (2009) Identification of potent pyrimidine inhibitors of phosphodiesterase 7 (PDE7) and their ability to inhibit T cell proliferation. *Bioorg Med Chem Lett* 19:1935–1938
- Hajjhussein H, Suvarna NU, Gremillion C, Chandler LJ, O'Donnell JM (2007) Changes in NMDA receptor-induced cyclic nucleotide synthesis regulated the age-dependent increase in PDE4A expression in primary cortical cultures. *Brain Res* 1149:58–68
- Haley JE, Malen PL, Chapman PF (1993) Nitric oxide synthase inhibitors block long term potentiation induced by weak but not strong tetanic stimulation at physiological brain temperatures in rat hippocampal slices. *Neurosci Lett* 160:85–88
- Han P, Zhu XY, Michaeli T (1997) Alternative splicing of the high affinity cAMP-specific phosphodiesterase (PDE7A) mRNA in human skeletal muscle and heart. *J Biol Chem* 272:16152–16157
- Han P, Werber J, Surana M, Fleischer N, Michaeli T (1999) The calcium/calmodulin-dependent phosphodiesterase PDE1C down-regulates glucose-induced insulin secretion. *J Biol Chem* 274:22337–22344
- Hartell NA (1996) Inhibition of cGMP breakdown promotes the induction of cerebellar long-term depression. *J Neurosci* 16:2881–2890
- Hebb ALO, Robertson HA, Denovan-Wright EM (2004) Striatal phosphodiesterase mRNA and protein levels are reduced in Huntington's Disease transgenic mice prior to the onset of motor symptoms. *Neuroscience* 123:967–981
- Hebb AL, Robertson HA, Denovan-Wright EM (2008) Phosphodiesterase 10A inhibition is associated with locomotor and cognitive deficits and increased anxiety in mice. *Eur Neuropsychopharmacol* 18:339–363

- Heikaus CC, Pandit J, Klevit RE (2009) Cyclic nucleotide binding GAF domains from phosphodiesterases: structural and mechanistic insights. *Structure* 17:1551–1557
- Holtmaat AJ, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW, Svoboda K (2005) Transient and persistent dendritic spines in the neocortex in vivo. *Neuron* 45:279–291
- Houslay MD (2001) PDE4 cAMP-specific phosphodiesterases. *Prog Nucleic Acid Res Mol Biol* 69:249–315
- Houslay MD (2010) Underpinning compartmentalised cAMP signalling through targeted cAMP breakdown. *Trends Biochem Sci* 35:91–100
- Houslay MD, Schafer P, Zhang KY (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. *Drug Discov Today* 10:1503–1519
- Houslay MD, Baillie GS, Maurice DH (2007) cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. *Circ Res* 100:950–966
- Hu H, McCaw EA, Hebb AL, Gomez GT, Denovan-Wright EM (2004) Mutant huntingtin affects the rate of transcription of striatum-specific isoforms of phosphodiesterase 10A. *Eur J Neurosci* 20:3351–3363
- Impey S, Mark M, Villacres EC, Poser S, Chavkin C, Storm DR (1996) Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. *Neuron* 16:973–982
- Itoh T, Tokumura M, Abe K (2004) Effects of rolipram, a phosphodiesterase 4 inhibitor, in combination with imipramine on depressive behavior, CRE-binding activity and BDNF level in learned helplessness rats. *Eur J Pharmacol* 498:135–142
- Juilfs DM, Fulle HJ, Zhao AZ, Houslay MD, Garbers DL, Beavo JA (1997) A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway. *Proc Natl Acad Sci USA* 94:3388–3395
- Kadoshima-Yamaoka K, Murakawa M, Goto M, Tanaka Y, Inoue H, Murafuji H, Nagahira A, Hayashi Y, Nagahira K, Miura K, Nakatsuka T, Chamoto K, Fukuda Y, Nishimura T (2009) ASB16165, a novel inhibitor for phosphodiesterase 7A (PDE7A), suppresses IL-12-induced IFN-gamma production by mouse activated T lymphocytes. *Immunol Lett* 122:193–197
- Kaulen P, Brüning G, Schneider HH, Sarter M, Baumgarten HG (1989) Autoradiographic mapping of a selective cyclic adenosine monophosphate phosphodiesterase in rat brain with the antidepressant [3H]rolipram. *Brain Res* 503:229–245
- Kelly MP, Brandon NJ (2009) Differential function of phosphodiesterase families in the brain: gaining insights through the use of genetically modified animals. *Prog Brain Res* 179:67–73
- Kelly MP, Logue SF, Brennan J, Day JP, Lakkaraju S, Jiang L, Zhong X, Tam M, Sukoff Rizzo SJ, Platt BJ, Dwyer JM, Neal S, Pulito VL, Agostino MJ, Grauer SM, Navarra RL, Kelley C, Comery TA, Murrills RJ, Houslay MD, Brandon NJ (2010) Phosphodiesterase 11A in brain is enriched in ventral hippocampus and deletion cause psychiatric disease related phenotypes. *Neuroscience* 107:8457–8462
- Kleppisch T (2009) Phosphodiesterases in the central nervous system. *Handb Exp Pharmacol* 191:71–92
- Kobayashi T, Gamanuma M, Sasaki T, Yamashita Y, Yuasa K, Kotera J, Omori K (2003) Molecular comparison of rat cyclic nucleotide phosphodiesterase 8 family: unique expression of PDE8B in rat brain. *Gene* 319:21–31
- Kotaleski JH, Blackwell Kim T (2010) Modelling the molecular mechanisms of synaptic plasticity using systems biology approaches. *Neuroscience* 11:239–249
- Kruuse C, Sergeid R, Tejvir S (2001) The role of cGMP hydrolyzing phosphodiesterase 1 and 5 in cerebral artery dilatation. *Eur J Pharmacol* 420:55–65
- Lee R, Wolda S, Moon E, Esselstyn J, Hertel C, Lerner A (2002) PDE7A is expressed in human B-lymphocytes and is up-regulated by elevation of intracellular cAMP. *Cell Signal* 14:277–284

- Lehericy S, Gerardin E (2002) Normal functional imaging of the basal ganglia. *Epileptic Disord* 4: S23–S30
- Lepage M, Ghaffar O, Nyberg L, Tulving E (2000) Prefrontal cortex and episodic memory retrieval mode. *Proc Natl Acad Sci USA* 97:506–511
- Li L, Yee C, Beavo JA (1999) CD3- and CD28-Dependent Induction of PDE7 Required for T Cell Activation. *Science* 283:848–851
- Li YF, Huang Y, Amsdell SL, Xiao L, O'Donnell JM, Zhang HT (2009) Antidepressant- and anxiolytic-like effects of the phosphodiesterase-4 (PDE4) inhibitor rolipram on behavior depend on cyclic AMP-response element binding protein (CREB)-mediated neurogenesis in the hippocampus. *Neuropsychopharmacology* 34:2404–2419
- Liebenberg N, Harvey BH, Brand L, Brink CB (2010) Antidepressant-like properties of phosphodiesterase type 5 inhibitors and cholinergic dependency in a genetic rat model of depression. *Behav Pharmacol* 21(5–6):540–547
- Lin CS (2004) Tissue expression, distribution, and regulation of PDE5. *Int J Impot Res* 16:8–10
- Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. *Neuron* 35:605–623
- Loughney K, Snyder PB, Uher L, Rosman GJ, Ferguson K, Florio VA (1999) Isolation and characterization of PDE10A, a novel human 3', 5'-cyclic nucleotide phosphodiesterase. *Gene* 234:109–117
- Loughney K, Taylor J, Florio VA (2005) Original research 3', 5'-Cyclic nucleotide phosphodiesterase 11A: localization in human tissues. *Int J Impot Res* 17:320–325
- Lu YF, Kandel ER, Hawkins RD (1999) Nitric oxide signaling contributes to late-phase LTP and CREB phosphorylation in the hippocampus. *J Neurosci* 19:10250–10261
- Lucassen H (2004) Pediatric hospice: a second home. *Pflege Z* 57:854–855
- Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 109:366–398
- MacKenzie SJ, Baillie GS, McPhee I, Bolger GB, Houslay MD (2000) ERK2 mitogen-activated protein kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterases. The involvement of COOH-terminal docking sites and NH₂-terminal UCR regions. *J Biol Chem* 275:16609–16617
- MacKenzie SJ, Baillie GS, McPhee I, MacKenzie C, Seamons R, McSorley T, Millen J, Beard MB, van Heeke G, Houslay MD (2002) Long PDE4 cAMP specific phosphodiesterases are activated by protein kinase A-mediated phosphorylation of a single serine residue in upstream conserved region 1 (UCR1). *Br J Pharmacol* 136:421–433
- Makhlouf A, Kshirsagar A, Niederberger C (2006) Phosphodiesterase 11: a brief review of structure, expression and function. *Int J Impot Res* 18:501–509
- Martinez SE, Beavo JA, Hol WG (2002) GAF domains: two-billion-year-old molecular switches that bind cyclic nucleotides. *Mol Interv* 2:317–323
- Masood A, Nadeem A, Mustafa SJ, O'Donnell JM (2008) Reversal of oxidative stress-induced anxiety by inhibition of phosphodiesterase-2 in mice. *J Pharmacol Exp Ther* 326: 369–379
- Masood A, Huang Y, Hajjhussein H, Xiao L, Li H, Wang W, Hamza A, Zhan CG, O'Donnell JM (2009) Anxiolytic effects of phosphodiesterase-2 inhibitors associated with increased cGMP signaling. *J Pharmacol Exp Ther* 331:690–699
- Matousovic K, Grande JP, Chini CC, Chini EN, Dousa TP (1995) Inhibitors of cyclic nucleotide phosphodiesterase isozymes type-III and type-IV suppress mitogenesis of rat mesangial cells. *J Clin Invest* 96:401–410
- Maurice DH, Haslam RJ (1990) Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol Pharmacol* 37:671–681
- McPhee I, Cochran S, Houslay MD (2001) The novel long PDE4A10 cyclic AMP phosphodiesterase shows a pattern of expression within brain that is distinct from the long PDE4A5 and short PDE4A1 isoforms. *Cell Signal* 13:911–918

- Mendelovic S, Doron A, Eilat E (1997) Short note: can depressive patients exploit the immune system for suicide. *Med Hypotheses* 49:445–446
- Mendelovic S, Doron A, Shoenfeld Y (1999) Depression and the immune system. *Harefuah* 136:88–91
- Menniti FS, Faraci WS, Schmidt CJ (2006) Phosphodiesterases in the CNS: targets for drug development. *Nat Rev Drug Discov* 5:660–670
- Menniti FS, Chappie TA, Humphrey JM, Schmidt CJ (2007) Phosphodiesterase 10A inhibitors: a novel approach to the treatment of the symptoms of schizophrenia. *Curr Opin Investig Drugs* 8:54–59
- Meyer MR, Angele A, Kremmer E, Kaupp B, Muller F (2000) A cGMP-signaling pathway in subset of olfactory sensory neurons. *PNAS* 97:10595–10600
- Millar JK, Pickard BS, Mackie S, James R, Christie S, Buchanan SR (2005) DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. *Science* 310:1187–1191
- Millar JK, Mackie S, Clapcote SJ, Murdoch H, Pickard BS, Christie S, Muir WJ, Blackwood DH, Roder JC, Houslay MD, Porteous DJ (2007) Disrupted in schizophrenia 1 and phosphodiesterase 4B: towards an understanding of psychiatric illness. *J Physiol* 584:401–405
- Miller CL, Oikawa M, Cai Y, Wojtovich AP, Nagel DJ, Xu X, Xu H, Florio V, Rybalkin SD, Beavo JA, Chen YF, Li JD, Blaxall BC, Abe J, Yan C (2009) Role of Ca²⁺/calmodulin-stimulated cyclic nucleotide phosphodiesterase 1 in mediating cardiomyocyte hypertrophy. *Circ Res* 105:956–964
- Miro X, Perez-Torres S, Palacios JM, Puigdomenech P, Mengod G (2001) Differential distribution of cAMP-specific phosphodiesterase 7A mRNA in rat brain and peripheral organs. *Synapse* 40:201–204
- Miró X, Pérez-Torres S, Puigdomènech P, Palacios JM, Mengod G (2002) Differential distribution of PDE4D splice variant mRNAs in rat brain suggests association with specific pathways and presynaptic localization. *Synapse* 45:259–269
- Miyamoto E (2006) Molecular mechanism of neuronal plasticity: induction and maintenance of long-term potentiation in the hippocampus. *J Pharmacol Sci* 100:433–442
- Molnar P, Gaal L (1992) Effect of different subtypes of cognition enhancers on longterm potentiation in the rat dentate gyrus in vitro. *Eur J Pharmacol* 215:17–22
- Monfort P, Muñoz MD, Kosenko E, Llansola M, Sánchez-Pérez A, Cauli O, Felipo V (2004) Sequential activation of soluble guanylate cyclase, protein kinase G and cGMP-degrading phosphodiesterase is necessary for proper induction of long-term potentiation in CA1 of hippocampus. Alterations in hyperammonemia. *Neurochem Int.* 2004 Nov;45(6):895–901
- Monti B, Berteotti C, Contestabile A (2006) Subchronic Rolipram Delivery Activates Hippocampal CREB and Arc, Enhances Retention and Slows Down Extinction of Conditioned Fear. *Neuropsychopharmacology* 31:278–286
- Mori F, Pérez-Torres S, De Caro R, Porzionato A, Macchi V, Beleta J, Gavaldà A, Palacios JM, Mengod G (2010) The human area postrema and other nuclei related to the emetic reflex express cAMP phosphodiesterases 4B and 4D. *J Chem Neuroanat* 40:36–42
- Mueller EM, Hofmann SG, Cherry JA (2010) The type IV phosphodiesterase inhibitor rolipram disturbs expression and extinction of conditioned fear in mice. *Neuropharmacology* 59:1–8
- Murata T, Shimizu K, Hiramoto K, Tagawa T, Murata T, Shimizu K, Hiramoto K, Tagawa T (2009) Phosphodiesterase 3 (PDE3): structure, localization and function. *Cardiovasc Hematol Agents Med Chem* 7:206–211
- Murdoch H, Mackie S, Collins DM, Hill EV, Bolger GB, Klussmann E, Porteous DJ, Millar JK, Houslay MD (2007) Isoform-selective susceptibility DISC1/phosphodiesterase-4 complexes to dissociation by elevated intracellular cAMP levels. *J Neurosci* 27:9513–9524
- Nagel DJ, Aizawa T, Jeon KI, Liu W, Mohan A, Wei H (2006) Role of nuclear Ca²⁺/calmodulin-stimulated phosphodiesterase 1A in vascular smooth muscle cell growth and survival. *Circ Res* 98:777–784

- Navakkode S, Sajikumar S, Frey JU (2005) Mitogen-activated protein kinase-mediated reinforcement of hippocampal early long-term depression by the type IV-specific phosphodiesterase inhibitor rolipram and its effect on synaptic tagging. *J Neurosci* 25:10664–10670
- Nguyen RV, Woo NH (2003) Regulation of hippocampal synaptic plasticity by cyclic AMP-dependent protein kinases. *Prog Neurobiol* 71:401–437
- Nikolaev VO, Gambaryan S, Engehardt S, Walter U, Lohse MJ (2005) Real-time monitoring of the PDE2 activity of live cells: hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis. *J Biol Chem* 280:1716–1719
- Numata S, Ueno S, Iga J, Song H, Nakataki M, Tayoshi S, Sumitani S, Tomotake M, Itakura M, Sano A, Ohmori TJ (2008) Positive association of the PDE4B (phosphodiesterase 4B) gene with schizophrenia in the Japanese population. *Psychiatr Res* 43:7–12
- O'Donnell JM, Zhang HT (2004) Antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE4). *Trends Pharmacol Sci* 25:158–163
- Obara Y, Nakahata N, Stork PJ (2009) cAMP signaling for ERK activation in neuronal cells. *Nippon Yakurigaku Zasshi* 133:63–68
- O'Conner MS, Steiner JM, Roussel AJ, Williams DA, Meddings JB, Pipers F, Cohen ND (2004) Evaluation of urine sucrose concentration for detection of gastric ulcers in horses. *Am J Vet Res* 65:31–39
- Oike Y, Hata A, Mamiya T, Kaname T, Noda Y, Suzuki M, Yasue H, Nabeshima T, Araki K, Yamamura K (1999) Truncated CBP protein leads to classical Rubinstein-Taybi syndrome phenotypes in mice: implications for a dominant-negative mechanism. *Hum Mol Genet* 8:387–396
- Oliveira RF, Terrin A, Di Benedetto G, Cannon RC, Koh W, Kim M, Zaccolo M, Blackwell KT (2010) The role of type 4 Phosphodiesterases in generating microdomains of cAMP: large scale stochastic simulations. *PLoS ONE* 5:e11725
- Olton DS (1983) Memory functions and the hippocampus. In: Seifert W (ed) *Neurobiology of the hippocampus*. Academic, London, pp 335–369
- Oray S, Majewska A, Sur M (2004) Dendritic spine dynamics are regulated by monocular deprivation and extracellular matrix degradation. *Neuron* 44:1021–1030
- Paizanis E, Hamon M, Lanfumey L (2007) Hippocampal neurogenesis, depressive disorders, and antidepressant therapy. *Neural Plast*. 2007:737–754
- Palmer D, Jimmo SL, Raymond DR, Wilson LS, Carter RL, Maurice DH (2007) Protein kinase A phosphorylation of human phosphodiesterase 3B promotes 14-3-3 protein binding and inhibits phosphatase-catalyzed inactivation. *J Biol Chem* 282:9411–9419
- Pause BM, Miranda A, Goder R, Aldenhoff JB, Ferstl R (2001) Reduced olfactory performance in patients with major depression. *J Psychiatr Res* 35:271–277
- Pérez-Torres S, Miró X, Palacios JM, Puigdoménech P, Mengod G (2001) PDE4 isozymes expression in human brain examined by in situ hybridization histochemistry and rolipram binding sites. Comparison with monkey and rat brains. *J Chem Neuroanat* 20:349–374
- Pelligrino DA, Wang Q (1998) Cyclic nucleotide crosstalk and the regulation of cerebral vasodilation. *Prog Neurobiol* 56:1–18
- Pereira A Jr, Furlan FA (2010) Astrocytes and human cognition: Modeling information integration and modulation of neuronal activity. *Neurobiology* 1046:1–16
- Pérez-Torres S, Miró X, Palacios JM, Cortés R, Puigdoménech P, Mengod G (2000) Phosphodiesterase type 4 isozymes expression in human brain examined by in situ hybridization histochemistry and [³H]rolipram binding autoradiography. Comparison with monkey and rat brain. *J Chem Neuroanat* 20:349–374
- Pérez-Torres S, Cortés R, Tolnay M, Probst A, Palacios JM, Mengod G (2003) Alteration of phosphodiesterase type 7 and 8 isozyme mRNA expression in Alzheimer's disease brains examined by in situ hybridization. *Exp Neurol* 182:322–334
- Pickard BS, Thomson PA, Christoforou A, Evans KL, Morris SW, Porteous DJ, Blackwood DH, Muir WJ (2007) The PDE4B gene confers sex-specific protection against schizophrenia. *Psychiatr Genet* 17:129–133

- Pilz RB, Broderick KE (2005) Role of cyclic GMP in gene regulation. *Front Biosci* 10:1239–1268
- Pollatos O, Kopietz R, Linn J, Albrecht J, Sakar V, Anzinger A, Schandry R, Wiesmann M (2007) Emotional stimulation alters olfactory sensitivity and odor judgment. *Chem Senses* 32: 583–589
- Prickaerts J, van Staveren WC, Sik A, Markerink-van Ittersum M, Niewöhner U, van der Staay FJ, Blokland A, de Vente J (2002) Effects of two selective phosphodiesterase type 5 inhibitors, sildenafil and vardenafil, on object recognition memory and hippocampal cyclic GMP levels in the rat. *Neuroscience* 113:351–361
- Prickaerts J, Sik A, van Staveren WC, Koopmans G, Steinbusch HW, van der Staay FJ, de Vente J, Blokland A (2004) Phosphodiesterase type 5 inhibition improves early memory consolidation of object information. *Neurochem Int* 45:915–928
- Puzzo D, Vitolo O, Trinchese F, Jacob JP, Palmeri A, Arancio O (2005) Amyloid-beta peptide inhibits activation of the nitric oxide/cGMP/cAMP-responsive element-binding protein pathway during hippocampal synaptic plasticity. *J Neurosci* 25:6887–6897
- Puzzo D, Staniszewski A, Deng SX, Privitera L, Leznik E, Liu S, Zhang H, Feng Y, Palmeri A, Landry DW, Arancio O (2009) Phosphodiesterase 5 inhibition improves synaptic function, memory, and amyloid-load in an Alzheimer's disease mouse model. *J Neurosci* 29:8075–8086
- Pyne NJ, Cooper ME, Houslay MD (1986) Identification and characterization of both the cytosolic and particulate forms of cyclic GMP-stimulated cyclic AMP phosphodiesterase from rat liver. *Biochem J* 234:325–334
- Quiroz JA, Machado-Vieira R, Zarate CA Jr, Manji HK (2010) Novel insights into lithium's mechanism of action: neurotrophic and neuroprotective effects. *Neuropsychobiology* 62:50–60
- Ramos BP, Birnbaum SG, Lindenmayer I, Newton SS, Duman RS, Arnsten AFT (2003) Dysregulation of protein kinase A signaling in the aged prefrontal cortex: new strategy for treating age-related cognitive decline. *Neuron* 40:835–845
- Ramos BP, Stark D, Verdusco L, van Dyck CH, Arnsten AF (2006) Alpha2A-adrenoceptor stimulation improves prefrontal cortical regulation of behavior through inhibition of cAMP signaling in aging animals. *Learn Mem* 13:770–776
- Rapoport M, van Reekum R, Mayberg H (2000) The role of the cerebellum in cognition and behavior: a selective review. *J Neuropsychiatry Clin Neurosci* 12:193–198
- Reinés A, Cereseto M, Ferrero A, Sifonios L, Podestá MF, Wikinski S (2008) Maintenance treatment with fluoxetine is necessary to sustain normal levels of synaptic markers in an experimental model of depression: correlation with behavioral response. *Neuropsychopharmacology* 33:1896–1908
- Reneerkens OA, Rutten K, Steinbusch HW, Blokland A, Prickaerts J (2009) Selective phosphodiesterase inhibitors: a promising target for cognition enhancement. *Psychopharmacology* 202: 419–443
- Reyes-Irisarri E, Pérez-Torres S, Mengod G (2005) Neuronal expression of cAMP-specific phosphodiesterase 7B mRNA in the rat brain. *Neuroscience* 132:1173–1185
- Rose GM, Hopper A, De Vivo M, Tehim A (2005) Phosphodiesterase inhibitors for cognitive enhancement. *Curr Pharm Des* 11:3329–3334
- Rutten K, Prickaerts J, Blokland A (2006) Rolipram reverses scopolamine-induced and time-dependent memory deficits in object recognition by different mechanisms of action. *Neurobiol Learn Mem* 85:132–138
- Rutten K, Prickaerts J, Hendrix M, van der Staay FJ, Sik A, Blokland A (2007) Time-dependent involvement of cAMP and cGMP in consolidation of object memory: studies using selective phosphodiesterase type 2, 4 and 5 inhibitors. *Eur J Pharmacol* 558:107–112
- Rutten K, Misner DL, Works M, Blokland A, Novak TJ, Santarelli L, Wallace TL (2008) Enhanced long-term potentiation and impaired learning in phosphodiesterase 4D-knockout (PDE4D) mice. *Eur J Neurosci* 28:625–632
- Rybalkin SD, Rybalkina I, Beavo JA, Bornfeldt KE (2002) Cyclic nucleotide phosphodiesterase 1C promotes human J. of Cardiovasc. Trans. Res. J. of Cardiovasc. Trans. Res. arterial smooth muscle cell proliferation. *Circ Res* 90:151–157

- Sasaki T, Yuasa K, Omori K (2000) Identification of human PDE7B, a cAMP specific phosphodiesterase. *Biochem Biophys Res Comm* 271:575–583
- Sasaki T, Kotera J, Omori K (2002) Novel alternative splice variants of rat phosphodiesterase 7B showing unique tissue-specific expression and phosphorylation. *Biochem J* 361:211–220
- Sasaki T, Kotera J, Omori K (2004) Transcriptional activation of phosphodiesterase 7B1 by dopamine D1 receptor stimulation through the cyclic AMP/cyclic AMP-dependent protein kinase/cyclic AMP-response element binding protein pathway in primary striatal neurons. *J Neurochem* 89:474–483
- Schaak S, Cayla C, Lymperopoulos A, Flordellis C, Cussac D, Denis C, Paris H (2000) Transcriptional down-regulation of the human alpha2C-adrenergic receptor by cAMP. *Mol Pharmacol* 58:821–827
- Schmidt CJ (2010) Phosphodiesterase inhibitors as potential cognition enhancing agents. *Curr Top Med Chem* 10:222–230
- Seeger TF, Bartlett B, Coskran TM, Culp JS, James LC, Krull DL, Lanfear J, Ryan AM, Schmidt CJ, Strick CA, Varghese AH, Williams RD, Wylie PG, Menniti FS (2003) Immunohistochemical localization of PDE10A in the rat brain. *Brain Res* 985:113–126
- Sette C, Iona S, Conti M (1994) The short-term activation of a rolipram-sensitive, cAMP-specific phosphodiesterase by thyroid-stimulating hormone in thyroid FRTL-5 cells is mediated by a cAMP-dependent phosphorylation. *J Biol Chem* 269:9245–9252
- Singh N, Parle M (2003) Sildenafil improves acquisition and retention of memory in mice. *Indian J Physiol Pharmacol* 47:318–324
- Suciak JA (2008) The role of phosphodiesterases in schizophrenia: therapeutic implications. *CNS Drugs* 22:983–993
- Suciak JA, McCarthy SA, Chapin DS, Fujiwara RA, James LC, Williams RD, Stock JL, McNeish JD, Strick CA, Menniti FS, Schmidt CJ (2006) Genetic deletion of the striatum-enriched phosphodiesterase PDE10A: evidence for altered striatal function. *Neuropharmacology* 51:374–385
- Suciak JA, Chapin DS, McCarthy SA, Martin AN (2007) Antipsychotic profile of rolipram: efficacy in rat and reduced sensitivity in mice deficient in the phosphodiesterase-4B (PDE4B) enzyme. *Psychopharmacology (Berl)* 192:415–424
- Suciak JA, McCarthy SA, Chapin DS, Martin AN (2008) Behavioral and neurochemical characterization of mice deficient in the phosphodiesterase-4B (PDE4B) enzyme. *Psychopharmacology* 197:115–126
- Sklar P, Gabriel SB, McInnis MG, Bennett P, Lim YM, Tsan G, Schaffner S, Kirov G, Jones I, Owen M, Craddock N, DePaulo JR, Lander ES (2002) Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. *Mol Psychiatry* 7:579–593
- Smith SJ, Cieslinski LB, Newton R, Donnelly LE, Fenwick PS, Nicholson AG, Barnes PJ, Barnette MS, Giembycz MA (2004) Discovery of BRL 50481 [3-(N, N-dimethylsulfonylamido)-4-methyl-nitrobenzene], a selective inhibitor of phosphodiesterase 7: in vitro studies in human monocytes, lung macrophages, and CD8+ T-lymphocytes. *Mol Pharmacol* 66:1679–1689
- Soderling SH, Bayuga SJ, Beavo JA (1999) Isolation and characterization of a dual-substrated phosphodiesterase gene family: PDE10A. *Proc Natl Acad Sci USA* 96:7071–7076
- Souness JE, Aldous D, Sargent C (2000) Immunosuppressive and anti-inflammatory effects of cyclic AMP phosphodiesterase (PDE) type 4 inhibitors. *Immunopharmacology* 47:127–162
- Ster J, de Bock F, Bertaso F, Abitbol K, Daniel H, Bockaert J, Fagni L (2009) Epac mediates PACAP-dependent long-term depression in the hippocampus. *J Physiol* 587:101–113
- Strick CA, James LC, Fox CB, Seeger TF, Menniti FS, Schmidt CJ (2010) Alterations in gene regulation following inhibition of the striatum-enriched phosphodiesterase, PDE10A. *Neuropharmacology* 58:444–451
- Surapitsitach J, Jeon KI, Yan C, Beavo JA (2007) Differential regulation of endothelial cell permeability by cGMP via phosphodiesterases 2 and 3. *Circ Res* 101:811–818

- Suvarna NU, O'Donnell JM (2002) Hydrolysis of N-methyl-D-aspartate receptor-stimulated cAMP and cGMP by PDE4 and PDE2 phosphodiesterases in primary neuronal cultures of rat cerebral cortex and hippocampus. *J Pharmacol Exp Ther* 302:249–256
- Takahashi M, Terwilliger R, Lane C, Mezes PS, Conti M, Duman RS (1999) Chronic antidepressant administration increases the expression of cAMP-specific phosphodiesterase 4A and 4B isoforms. *J Neurosci* 19:610–618
- Tanis KQ, Duman RS (2007) Intracellular signaling pathways pave roads to recovery for mood disorders. *Ann Med* 39:531–544
- Taylor JR, Birnbaum SG, Ubriani R, Arnsten AFT (1999) Activation of protein kinase A in prefrontal cortex impairs working memory performance. *J Neurosci* 19:RC23
- Taylor RE, Shows KH, Zhao Y, Pittler SJ (2001) A PDE6A promoter fragment directs transcription predominantly in the photoreceptor. *Biochem Biophys Res Commun* 282:543–547
- Teng FY, Tang BL (2006) Axonal regeneration in adult CNS neurons—signaling molecules and pathways. *J Neurochem* 96:1501–1508
- Thompson PE, Manganiello V, Degerman E (2007) Re-discovering PDE3 inhibitors – new opportunities for a long neglected target. *Curr Top Med Chem* 7:421–436
- Titus SA, Li X, Southall N, Lu J, Inglese J, Brasch M, Austin CP, Zheng W (2008) A cell-based PDE4 assay in 1536-well plate format for high-throughput screening. *J Biomol Screen* 13:609–618
- Torrás-Llort M, Azorin F (2003) Functional characterization of the human phosphodiesterase 7A1 promoter. *Biochem J* 373:835–843
- Uthayathas S, Karuppagounder SS, Thrash BM, Parameshwaran K, Suppiramaniam V, Dhanasekaran M (2007) Versatile effects of sildenafil: recent pharmacological applications. *Pharmacol Rep* 59:150–163
- Valera E, Sánchez-Martín FJ, Ferrer-Montiel AV, Messeguer A, Merino JM (2008) NMDA-induced neuroprotection in hippocampal neurons is mediated through the protein kinase A and CREB (cAMP-response element-binding protein) pathway. *Neurochem Int* 58:148–154
- Van der Staay FJ, Rutten K, Bärfacker L, Devry J, Erb C, Heckroth H, Karthaus D, Tersteegen A, van Kampen M, Blokland A, Prickaerts J, Reymann KG, Schröder UH, Hendrix M (2008) The novel selective PDE9 inhibitor BAY 73-6691 improves learning and memory in rodents. *Neuropharmacology* 55:908–918
- van Donkelaar EL, Rutten K, Blokland A, Akkerman S, Steinbusch HW, Prickaerts J (2008) Phosphodiesterase 2 and 5 inhibition attenuates the object memory deficit induced by acute tryptophan depletion. *Eur J Pharmacol* 600:98–104
- Van Staveren WCG, Markerink-Van Ittersum M, Steinbusch HWM, De Vente J (2001) The effect of phosphodiesterase inhibition on cyclic GMP and cyclic AMP accumulation in the hippocampus of the rat. *Brain Res* 888:275–286
- Van Staveren WC, Steinbusch HW, Markerink-van Ittersum M, Repaske DR, Goy MF, Kotera J, Omori K, Beavo JA, De Vente J (2003) mRNA expression patterns of the cGMP-hydrolyzing phosphodiesterases types 2, 5, and 9 during development of the rat brain. *J Comp Neurol* 467:566–580
- Vecsey CG, Baillie GS, Jaganath D, Havekes R, Daniels A, Wimmer M, Huang T, Brown KM, Li XY, Descalzi G, Kim SS, Chen T, Shang YZ, Zhuo M, Houslay MD, Abel T (2009) Sleep deprivation impairs cAMP signalling in the hippocampus. *Nature* 461:1122–1125
- Viana RJ, Fonseca MB, Ramalho RM, Nunes AF, Rodrigues CM (2010) Organelle stress sensors and cell death mechanisms in neurodegenerative diseases. *CNS Neurol Disord Drug Targets* 9(6):679–692
- Wachtel H (1982) Characteristic behavioural alterations in rats induced by rolipram and other selective adenosine cyclic 3', 5'-monophosphate phosphodiesterase inhibitors. *Psychopharmacol (Berl)* 77:309–316
- Weeks JL, Zoraghi R, Francis SH, Corbin JD (2007) N-Terminal domain of phosphodiesterase-11A4 (PDE11A4) decreases affinity of the catalytic site for substrates and tadalafil, and is involved in oligomerization. *Biochemistry* 46:10353–10364

- Whitaker CM, Wei H (2009) An alternate cAMP pathway Epac promotes hippocampal long-term depression. *J Physiol* 587:3067–3068
- Wong ML, Whelan F, Deloukas P, Whittaker P, Delgado M, Cantor RM, McCann SM, Licinio J (2006) Phosphodiesterase genes are associated with susceptibility to major depression and antidepressant treatment response. *Proc Natl Acad Sci USA* 103:15124–15129
- Woolfrey KM, Srivastava DP, Photowala H, Yamashita M, Barbolina MV, Cahill ME, Xie Z, Jones KA, Quilliam LA, Prakriya M, Penzes P (2009) Epac2 induces synapse remodeling and depression and its disease-associated forms alter spines. *Nat Neurosci* 12:1275–1284
- Wu AY, Tang XB, Martinez SE, Ikeda K, Beavo JA (2004) Molecular determinants for cyclic nucleotide binding to the regulatory domains of phosphodiesterase 2A. *J Biol Chem* 279:37928–37938
- Wunder F, Tersteegen A, Rebmann A, Erb C, Fahrig T, Hendrix M (2005) Characterization of the first potent and selective PDE9 inhibitor using a cGMP reporter cell line. *Mol Pharmacol* 68:1775–1781
- Xie Z, Adamowicz WO, Eldred WD, Jakowski AB, Kleiman RJ, Morton DG, Stephenson DT, Strick CA, Williams RD, Menniti F (2006) Cellular and subcellular localization of PDE10A, a striatum-enriched phosphodiesterase. *Neuroscience* 139:597–607
- Yang G, McIntyre KW, Townsend RM, Shen HH, Pitts WJ, Dodd JH, Nadler SG, McKinnon M, Watson AJ (2003) Phosphodiesterase 7A-deficient mice have functional T cells. *J Immunol* 171:6414–6420
- Yuasa K, Kanoh Y, Okumura K, Omori K (2001) Genomic organization of the human phosphodiesterase PDE11A gene, Evolutionary relatedness with other PDEs containing GAF domains. *Eur J Biochem* 268:168–178
- Zaccolo M, Movsesian MA (2007) cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res* 100:1569–1578
- Zhan Y, Zhang HT, O'Donnell JM (2003) Antidepressant-induced increase in high-affinity rolipram binding sites in rat brain: dependence on noradrenergic and serotonergic function. *J Pharmacol Exp Ther* 307:246–253
- Zhang HT (2009) Cyclic AMP-specific phosphodiesterase-4 as a target for the development of antidepressant drugs. *Curr Pharm Des* 15:1687–1698
- Zhang HT, O'Donnell JM (2000) Effects of rolipram on scopolamine-induced impairment of working and reference memory in the radial-arm maze tests in rats. *Psychopharmacology* 150:311–316
- Zhang HT, Crissman AM, Dorairaj NR, Chandler LJ, O'Donnell JM (2000) Inhibition of cyclic AMP phosphodiesterase (PDE4) reverses memory deficits associated with NMDA receptor antagonism. *Neuropsychopharmacology* 23:198–204
- Zhang HT, Huang Y, Jin SL, Frith SA, Suvarna N, Conti M, O'Donnell JM (2002a) Antidepressant-like profile and reduced sensitivity to rolipram in mice deficient in the PDE4D phosphodiesterase enzyme. *Neuropsychopharmacology* 27:587–595
- Zhang R, Wang Y, Zhang L, Zhang Z, Tsang W, Lu M, Zhang L, Chopp M (2002b) Sildenafil 1 (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke* 33:2675–2680
- Zhang HT, Zhao Y, Huang Y, Dorairaj NR, Chandler LJ, O'Donnell JM (2004) Inhibition of the phosphodiesterase 4 (PDE4) enzyme reverses memory deficits produced by infusion of the MEK inhibitor U0126 into the CA1 subregion of the rat hippocampus. *Neuropsychopharmacology* 29:1432–1439
- Zhang HT, Huang Y, Suvarna NU, Deng C, Crissman AM, Hopper AT, De Vivo M, Rose GM, O'Donnell JM (2005) Effects of the novel PDE4 inhibitors MEM1018 and MEM1091 on memory in the radial-arm maze and inhibitory avoidance tests in rats. *Psychopharmacology* 179:613–619
- Zhang L, Zhang Z, Zhang RL, Cui Y, LaPointe MC, Silver B, Chopp M (2006) Tadalafil, a long-acting type 5 phosphodiesterase isoenzyme inhibitor, improves neurological functional recovery in a rat model of embolic stroke. *Brain Res* 1118:192–198

- Zhang HT, Huang Y, Masood A, Stolinski LR, Li Y, Zhang L, Dlaboga D, Jin SL, Conti M, O'Donnell JM (2008) Anxiogenic-like behavioral phenotype of mice deficient in phosphodiesterase 4B (PDE4B). *Neuropsychopharmacology* 33:1611–1623
- Zhao J, Harada N, Kurihara H, Nakagata N, Okajima K (2010) Cilostazol improves cognitive function in mice by increasing the production of insulin-like growth factor-I in the hippocampus. *Neuropharmacology* 58:774–783

Therapeutic Potential of Phosphodiesterase Inhibitors in Parasitic Diseases

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Abstract Protozoan parasites of the order kinetoplastida are the causative agents of three of the world's most important neglected human diseases: African trypanosomiasis, American trypanosomiasis, and leishmaniasis. Current therapies are limited, with some treatments having serious and sometimes lethal side effects.

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The growing number of cases that are refractory to treatment is also of concern. With few new drugs in development, there is an unmet medical need for new, more effective, and safer medications. Recent studies employing genetic and pharmacological techniques have begun to shed light on the role of the cyclic nucleotide phosphodiesterases in the life cycle of these pathogens and suggest that these important regulators of cyclic nucleotide signaling may be promising new targets for the treatment of parasitic diseases.

Keywords Crystal structure · Kinetoplastid · Cyclic AMP · Cyclic nucleotide phosphodiesterase · PDE inhibitor · RNA interference

Abbreviations

AC	Adenylate cyclase
cAMP	Cyclic 3',5' adenosine monophosphate
cGMP	Cyclic 3',5' guanosine monophosphate
CL	Cutaneous leishmaniasis
DAPI	4,6'-diamidino-2-phenylindole
EPAC	Exchange protein activated by cAMP
HAT	Human African trypanosomiasis
IBMX	Isobutyl-1-methylxanthine
MCL	Mucocutaneous leishmaniasis
PDE	Cyclic nucleotide phosphodiesterase
PKA	Protein kinase A
RNAi	RNA interference
SIF	Stumpy inducing factor
VL	Visceral leishmaniasis

1 Introduction

Millions of people worldwide suffer from diseases caused by vector-borne protozoan parasites. The main focus of this chapter is the causative agent of three of the world's most important neglected diseases: African trypanosomiasis (sleeping sickness), American trypanosomiasis (Chagas disease), and the leishmaniasis. In particular, the current state of the art of the characterization and elucidation of the functional role of the cyclic nucleotide phosphodiesterases (PDEs) in the life cycle of these parasites and their potential as targets for new therapies are discussed.

1.1 *Kinetoplastida: Life Cycle and Disease*

Trypanosoma and Leishmania parasites are the causative agents of trypanosomiasis and leishmaniasis, respectively; both are protozoan unicellular parasites belonging to

the order kinetoplastida, so called because of a DNA-containing organelle within the mitochondria known as the kinetoplast. In addition to the kinetoplast, these parasites are characterized by the presence of a single flagellum, an organelle that plays a key role in several critical cell functions, such as motility, cell division, morphogenesis, attachment to vector- and host-cell surfaces, immune evasion, and consequently, the viability and pathogenic potential of the parasite (Hunger-Glaser and Seebeck 1997; Broadhead et al. 2006). The life cycle of the kinetoplastid parasites is complex, comprising both insect and human stages that involve morphological and metabolic adaptation of the parasite to enable survival and propagation in the differing environments encountered (Black and Seed 2001; Farrell 2002; Tyler and Miles 2002). The life cycle of each parasite is shown in Fig. 1.

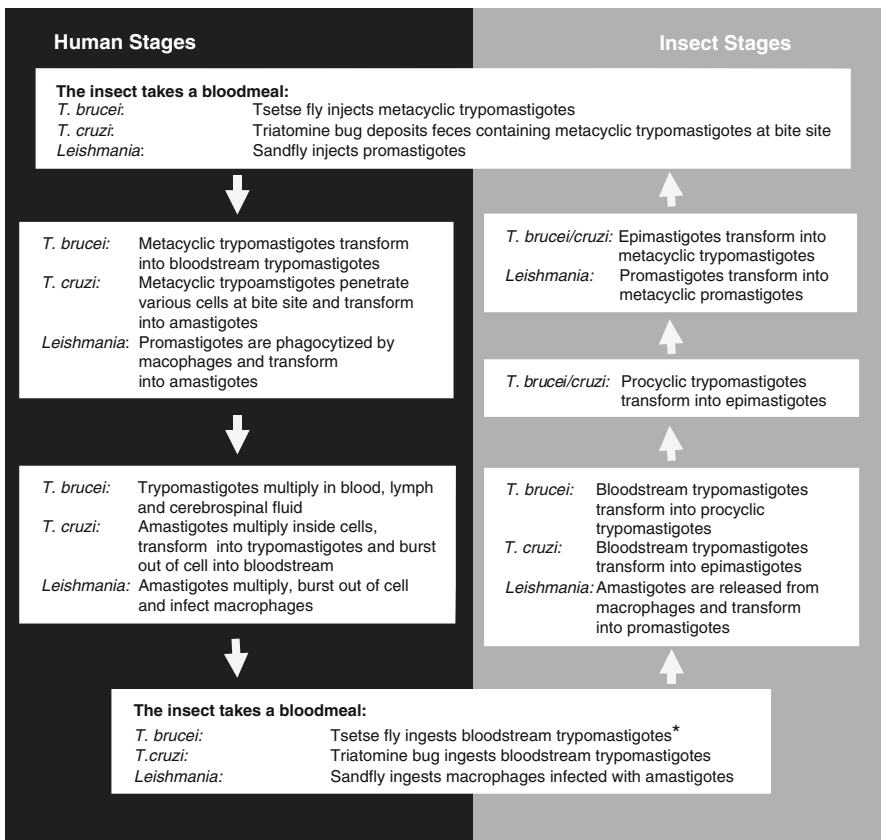


Fig. 1 Life cycles of *T. brucei*, *T. cruzi*, and *Leishmania*. *Bloodstream trypomastigotes exist in two forms: the long slender proliferating form and the short stumpy nonproliferating form that is preadapted for survival in the tsetse fly

1.1.1 *Trypanosoma brucei*

Human African trypanosomiasis (HAT) is caused by the kinetoplastid *Trypanosoma brucei* (*T. brucei*) and transmitted to humans by the bite of the blood-sucking tsetse fly. Two subspecies, *T. brucei rhodesiense* and *T. brucei gambiense*, are responsible for acute and chronic forms of HAT, known as East and West African trypanosomiasis, respectively. A third subspecies, *T. brucei brucei*, causes animal trypanosomiasis, also known as nagana, as do the closely related species *T. congolense*, *T. evansi*, and *T. vivax*. The first sign of infection may be the formation of a chancre at the bite site. During the first stage, or hemolymphatic stage of the disease, trypanosomes invade the host bloodstream. The accompanying symptoms are often nonspecific (fever, headaches, enlarged lymph nodes, joint pain, and itching). Indeed, patients may be asymptomatic, making early diagnosis difficult. The disease is characterized by waves of parasitemia, during which parasite numbers peak and then decline almost to zero. Parasite survival is ensured due to surface coat antigenic variants that are not recognized by the host immune system. Each successive wave of parasitemia represents a new antigenic variant. In the second phase, or neurological phase of the disease, which may occur within weeks or months (East African trypanosomiasis), or years later (West African trypanosomiasis), the trypanosomes invade the host central nervous system. Symptoms during this phase include pancarditis, meningoencephalitis, confusion, sensory disturbance, disruption of the sleep–wake cycle, loss of coordination, and coma and are fatal if not treated. HAT is endemic in 36 sub-Saharan countries: there are 50 million people at risk of contracting HAT and approximately 30,000 HAT-related deaths per year (Stuart et al. 2008; Brun et al. 2010).

1.1.2 *Trypanosoma cruzi*

American trypanosomiasis is caused by the kinetoplastid *Trypanosoma cruzi* (*T. cruzi*) and is mainly transmitted in the feces of the blood-sucking triatomine bugs. Other routes of infection are via blood transfusion and vertical transmission from mother to fetus. Acute phase symptoms are generally mild and nonspecific and often include a swelling at the site of infection, known as a chagoma or Romaña's sign. Following the acute phase, the disease generally enters a life-long latent phase. In approximately 30% of cases, however, life-threatening symptoms develop decades after the initial infection, most often as complications of the heart or gut. The disease is endemic in 19 Central and South American countries and is estimated to affect 8–11 million people (Stuart et al. 2008). Chagas disease is the leading cause of heart failure in Central and South America and the cause of approximately 14,000 deaths annually (Barrett et al. 2003; Stuart et al. 2008). Immigration of large populations of Latin Americans to other countries has spread the risk of *T. cruzi* infection to regions outside Central and South America. In recent years, *T. cruzi* contamination has been identified in blood banks in the USA and other countries following blood donations by individuals unaware they were infected.

1.1.3 *Leishmania* Species

The human leishmaniasis are caused by at least 21 *Leishmania* species. The parasite is transmitted to humans by the bite of female sandflies (*Lutzomyia* spp and *Phlebotomus* spp) and, depending on the infecting species, may cause cutaneous (CL), mucocutaneous (MCL), or visceral (VL) leishmaniasis. CL manifests as localized lesions on exposed regions of the skin, which may or may not be self-healing. MCL is a complication of CL that results in extensive destruction of the mucous membranes of the nose, mouth, or throat. Although not lethal, CL and, in particular, MCL can be severely disfiguring and the cause of social stigmatization. VL affects the internal organs, including the liver, spleen, lymph nodes, and bone marrow. Symptoms include fever, enlarged liver and spleen, anemia, anorexia, and weight loss and are fatal if not treated. The leishmaniasis occur in five continents, are endemic in 88 countries, and affect 12 million people worldwide (Stuart et al. 2008). CL occurs predominantly in the Middle East, MCL in Central and South America, and VL mostly in Bangladesh, Brazil, India, Nepal, and Sudan. VL kills approximately 51,000 people every year and its occurrence is increasing in South-western Europe, where it has emerged as an opportunistic infection of the immunocompromised, particularly those with HIV (Alvar et al. 2008). The increased incidence of the disease in this region has been linked to transmission caused by infected drug users sharing needles.

1.2 *Therapies Currently in Clinical Use or in Development*

Despite the fact that approximately 500 million people worldwide are at risk from kinetoplastid-related diseases, advances in the development of new, safer, and more efficacious therapies have lagged far behind those of drugs for more profitable indications. According to the Drugs for Neglected Diseases initiative, of the 1,556 new drugs approved between 1975 and 2004, only 6 (0.4%) were developed for the most neglected diseases (i.e., HAT, Chagas disease, and leishmaniasis) (Chirac and Torreele 2006). Being predominantly afflictions of the world's poorest populations, there is little or no financial incentive for development of new drugs. Consequently, many treatments in use today consist of drugs that were introduced over 50 years ago. These drugs are far from ideal, with major problems including toxicity, resistance, difficult administration regimens, and prohibitively high cost (Stuart et al. 2008).

The mainstay treatment for early-stage HAT consists of two drugs, suramin and pentamidine, used to treat the East and West African forms of the disease, respectively. Neither drug can cross the blood–brain barrier and consequently they are not effective against late-stage disease. Two drugs, melarsoprol and eflornithine, are currently used for late-stage disease. Melarsoprol, an arsenic-containing compound, is highly toxic and causes death in 1 in 20 patients due to encephalopathy. Eflornithine is only effective for the West African disease and its use is predominantly

restricted to cases refractory to melarsoprol because of its high cost and lengthy administration regime (Delespaux and De Koning 2007).

The two treatments for acute American trypanosomiasis are nifurtimox and benznidazole, neither of which is particularly effective against chronic disease (Urbina 2010). Although taken orally, the lengthy treatment course of 30–60 days together with dose and time-related side effects (e.g., anorexia, weight loss, nausea and vomiting, skin rashes, and neuropathy) contributes to a high rate of noncompliance (Castro et al. 2006).

First-line therapies for the leishmaniases (CL and VL) are the pentavalent antimonials (Croft and Coombs 2003), except in India where these drugs are ineffective against VL due to the emergence of drug-resistant strains (Olliaro et al. 2005). In such cases, three drugs are available: lipid formulations of amphotericin B (high cost can be restrictive), paromomycin (more side effects than amphotericin B), and miltefosine (contraindicated in pregnancy due to its teratogenicity) (Olliaro et al. 2005).

There are currently no new drugs in clinical development for Chagas disease or leishmaniasis. Two clinical trials have recently been conducted for HAT, one for an orally available derivative of pentamidine (DB289) and the other for a nifurtimox-eflornithine combination therapy (NECT): the development of DB289 has now been halted due to late renal toxicity (Paine et al. 2010), but NECT appears to offer some improvement on the standard eflornithine monotherapy (Priotto et al. 2009).

1.3 Cyclic Nucleotide Signaling in the Kinetoplastid Parasites

A role for cAMP signaling in the cell biology and virulence of kinetoplastids was first suggested by Strickler and Patton (Strickler and Patton 1975) for the rodent trypanosome *T. lewisi*, by Mancini and Patton (Mancini and Patton 1981) for *T. brucei*, and by Walter et al. (Walter et al. 1978) for *L. donovani*. However, progress in this area has been slow and more than 30 years later many of the fundamental questions remain unanswered (Seebeck et al. 2004; Kunz et al. 2009b). A major impediment was the implicit assumption by many researchers that cAMP signaling in kinetoplastids was organized according to the accepted paradigms in higher organisms at that time, most notably mammals. As a consequence, many experiments were designed and results interpreted with the mammalian models in mind, only to be followed by the slow realization that most of what seemed to be “highly conserved” components of eukaryotic cAMP signaling networks were not, in fact, conserved in the kinetoplastids.

For example, kinetoplastid class II adenylate cyclases (AC) are structurally distinct from mammalian class I ACs. Mammalian ACs contain several transmembrane domains and are regulated by G-protein-coupled receptors. Kinetoplastid ACs contain a single transmembrane region, flanked on one side by an extracellular

domain that may act as a receptor, and on the other by an intracellular catalytic domain that shares homology with the catalytic domain of the class I ACs. The kinetoplastid ACs are not regulated by G proteins, the absence of G-protein-coupled receptors and heterotrimeric G proteins was confirmed when the complete sequence of the first kinetoplastid genome (*T. brucei*) became available in 2005 (Berriman et al. 2005). Kinetoplastid AC activity is unaffected by forskolin or cholera toxin and neither agonists nor antagonists have yet been identified. With regard to the downstream effectors of cAMP, while no counterparts for the mammalian cyclic nucleotide-gated channels or exchange proteins activated by cAMP (EPAC) have been identified in kinetoplastids, protein kinase A (PKA) is present in a fairly conserved form in the trypanosomatid genomes (genes for one regulatory and three catalytic subunits have been identified) (Siman-Tov et al. 1996; Huang et al. 2002, 2006). Although early studies suggested kinetoplastid PKA activity to be unresponsive to cAMP (Shalaby et al. 2001), more recent studies have revealed the presence of a cAMP-responsive PKA activity in *T. cruzi* (Huang et al. 2006). Moreover, *T. cruzi* cAMP-PDE, TcPDEC2, has been identified as an interaction partner of the catalytic subunit of PKA from *T. cruzi* (TcPKAc) and the recombinant TcrPDEC2 is phosphorylated by PKA (Huang et al. 2006). Another important component that is substantially conserved between mammals and kinetoplastids is the cyclic nucleotide phosphodiesterase (PDE). There are four genetically distinct kinetoplastid PDE families and recent studies suggest that the enzyme plays a vital role in the regulation and compartmentalization of cAMP signaling (Zoraghi and Seebeck 2002; Kunz et al. 2006; Oberholzer et al. 2007). These PDEs are pharmacologically distinct from mammalian PDEs as none of the mammalian PDE inhibitors tested to date have shown significant activity (Zoraghi et al. 2001; Laxman et al. 2006; Johner et al. 2006). Interestingly, an analysis of the flagellar proteome of *T. brucei* has revealed the presence of several enzymes involved in cAMP signaling, including two cAMP-PDEs, adenylate kinases, and putative cAMP-binding proteins (Broadhead et al. 2006). Experimental evidence points to the PDEs being part of a larger signaling complex associated with the paraflagellar rod, a highly organized filamentous structure running parallel to the axoneme (Oberholzer et al. 2007).

Although the results of early studies employing the use of mammalian PDE inhibitors or class I AC agonists, now known to be ineffective against their kinetoplastid counterparts, must be reassessed, changes in cAMP levels have been observed to precede kinetoplastid transformation and differentiation. *T. brucei* bloodstream trypomastigotes exist in two forms, known as the long slender proliferating form and the short stumpy nonproliferating form; the latter is preadapted for survival in the tsetse fly (see Fig. 1). Transformation from the long slender to short stumpy form is induced at a critical density, corresponding to the peak of parasitemia in the mammalian host. Cyclic AMP levels in the long slender form at the peak of parasitemia were found to be five times higher than that in the short stumpy form (Reed et al. 1985). In *T. cruzi*, transformation of epimastigotes into metacyclic trypomastigotes is also preceded by a threefold increase in cAMP (Rangel-Aldao et al. 1988). In a pleomorphic *T. brucei* strain, a quorum-sensing

molecule called stumpy inducing factor (SIF) promotes cell cycle arrest in G1/G0 phase and induces differentiation of trypomastigotes from the long slender form into the short stumpy form. Treatment of the long slender form with SIF was accompanied by an increase in cAMP, suggesting that this may be the trigger for differentiation (Vassella et al. 1997). The link between increased cAMP and its effects on transformation and proliferation appeared to be supported by studies using membrane-permeable analogues of cAMP, assumed at the time to be resistant to hydrolysis. For example, pCPT-cAMP mimicked the effect of SIF to induce differentiation of *T. brucei* trypomastigotes from the long slender to the short stumpy form (Vassella et al. 1997; Breidbach et al. 2002). In *Leishmania*, exposure to dibutyryl cAMP decreased *L. tropica* promastigote proliferation and induced transformation of *L. donovani* amastigotes to promastigotes (Walter et al. 1978). However, a recent study has demonstrated that the membrane-permeable cAMP analogue, 8-pCPT-cAMP is, in fact, efficiently hydrolyzed to 8-pCPT-5'AMP by the kinetoplastid PDEs (Laxman et al. 2006). Comparison of the effect of the cAMP analogue and its corresponding AMP and adenosine analog breakdown products suggested that slender to stumpy transformation was mediated by the cAMP hydrolysis products 5'AMP and adenosine and not by cAMP per se (Laxman et al. 2006). These studies were conducted on a monomorphic strain of *T. brucei* that has lost the ability to transform from the slender to the stumpy form in vivo. Monomorphic strains differ from pleomorphic strains in their cAMP responses; unlike pleomorphic strains they do not show an increase in cAMP following exposure to SIF (Vassella et al. 1997). Thus, while many questions remain about the role of cAMP in the transformation and differentiation of *T. brucei*, a consensus is emerging that cyclic nucleotide signaling does play a role in kinetoplastid life cycle progression. For more comprehensive reviews on cAMP signaling in kinetoplastids, please refer to Seebeck et al. (2004), Laxman and Beavo (2007) and Kunz et al. (2009b).

While the absence of effective pharmacological tools has been a drawback, this limitation has partly been overcome with the use of gene knockdown via RNA interference (RNAi) techniques and recent studies have begun to provide valuable insights on the role of cAMP in the cell biology of these parasites (see Sect. 2).

2 Kinetoplastid Phosphodiesterases

Although the presence of cAMP-PDEs in kinetoplastid parasites has been known for at least three decades (Walter 1974), the molecular analysis and exploration of these enzymes as potential drug targets has only recently begun.

2.1 Primary Structure

The genomes of all currently sequenced kinetoplastids (*T. brucei*, *T. cruzi*, *T. congolense*, *T. vivax*, *Leishmania major*, *L. infantum*, *L. braziliensis*, and

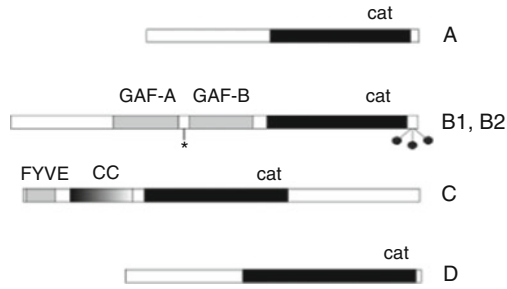


Fig. 2 Schematic representation of the four kinetoplast PDE families. *Cat* class 1 catalytic domain, *GAF-A*, *GAF-B* GAF domains of PDEB1 and PDEB2, *FYVE* N-terminal FYVE domain of PDEC, *CC* coiled-coiled domain, *asterisk* predicted conserved PKA phosphorylation site between the GAF domains, *black dots* phosphorylated serine residues at the C-terminus of TbrPDEB1

L. tarentolae; <http://tritrypdb.org/tritrypdb/>) contain the same set of four class I PDEs (Fig. 2 and Table 1). PDEA, PDEC, and PDED are all single-copy genes in *T. brucei* and *Leishmania* (see Table 1), with *T. cruzi* representing a special case. The *T. cruzi* CL Brener strain that was used for genome sequencing is a hybrid, so that many single-copy genes are actually present in two versions that reflect their different ancestry (Weatherly et al. 2009). For PDEB, all the above genomes contain two tandemly linked, very similar, but nonidentical copies. The PDEs coded by these two genes, PDEB1 and PDEB2, exhibit distinct subcellular locations (see below). A unified nomenclature for the kinetoplast PDEs has been proposed (Kunz et al. 2006); an updated version is given in Table 1.

PDEA. TbrPDEA was the first trypanosomatid PDE to be cloned, expressed, and characterized (Kunz et al. 2004). Subsequently, orthologues of TbrPDEA were identified in all kinetoplastid genomes. They are all single-copy genes, with the exception of *T. cruzi* where two copies are present and share between 40 and 60% amino acid sequence identity. The *T. brucei* gene (formerly designated TbPDE1) was functionally identified as a cAMP-PDE by complementation screening of a *T. brucei* cDNA library in the yeast *Streptomyces cerevisiae* (Kunz et al. 2004): it is a single-copy gene located on chromosome 10, which codes for a protein of 620 amino acids. Constitutively low levels of mRNA are present both in bloodstream and in procyclic forms. The class 1 catalytic domain of TbrPDEA is situated in the C-terminal region (M₂₈₅-Q₆₁₈) and exhibits cAMP-selectivity, with an unusually high K_m for cAMP of >600 μM . The enzyme is fully resistant to most commercial PDE inhibitors, but is inhibited by sildenafil and trequinsin with approximate K_i values of 1.0–2.5 μM , respectively. Very similar results were also obtained with its *T. cruzi* homologue, TcrPDEA1, which exhibits a K_m for cAMP of 190 μM , is not affected by cGMP, and complements a PDE-deficient yeast strain (Alonso et al. 2007).

For *L. major*, the gene for LmjPDEA was also shown to complement a PDE-deficient strain of *S. cerevisiae* (Johner et al. 2006). A common observation for the

Table 1 Updated nomenclature of kinetoplastid PDEs (human pathogens only)

Enzyme family		Older designations	Protein and gene identification	Number	References
PDEA	TbrPDEA	TbPDE1	Q388U6 ^(EMBL) Tb10.389.0510 ^(TriTryp)	10	Kunz et al. (2004), Gong et al. (2001)
–	TcrPDEA-P	–	Q309F4 Tc00.1047053511269.40	40 P	–
–	TcrPDEA-S	TcrPDEA1	Q4E006 Tc00.1047053509805.20	40 S	Alonso et al. (2007)
–	LmjPDEA	–	Q6S998 LmjF18.1090	18	Johner et al. (2006)
–	LinPDEA	–	A4HXY3 LinJ18.1100	18	–
–	LbrPDEA	–	A4H9L9 LbrM18.0980	18	–
–	–	–	–	–	–
PDEB	TbrPDEB1	TbPDEC	Q8WQX9 Tb09.160.3590	9	Zoraghi and Seebeck (2002), Oberholzer et al. (2007)
–	TcrPDEB1	–	Q4E0Y6 Tc00.1047053508277.100	7 S	Diaz-Benjumea et al. (2006)
–	LmjPDEB1	–	Q6S996 LmjF15.1480 ^a	15	Johner et al. (2006)
–	LinPDEB1	–	A4HWN7 LinJ15_V3.1550	15	–
–	LbrPDEB1	–	A4H8B6 LbrM15.1080	15	–
–	–	–	–	–	–
–	TbrPDEB2a	TbPDE2B	Q48F32 Tb09.160.3630	9	Rascon et al. (2002), Oberholzer et al. (2007)
–	TbrPDEB2b ^b	–	Q48F32 ^b Tb09.160.3630	9	Kunz et al. (2009a)
–	TcrPDEB2-P	–	Q4CQR0 Tc00.1047053509943.20	7 P	–
–	TcrPDEB2-S	TcPDE1	Q4E0Y5 Tc00.1047053508277.110	7 S	D'Angelo et al. (2004)
–	LmjPDEB2	–	Q6S997 n.a.	n.a.	–
–	LinPDEB2	–	A4HWN6 LinJ15_V3.1540	15	–
–	LbrPDEB2	–	A4H893 LbrM15.0880	15	–
–	–	–	–	–	–
PDEC	TbrPDEC	–	Q75ZK5 Tb03.27C5.640	3	–
–	TcrPDEC-P ^c	–	n.a. Tc00.1047053508153.520	36-P	–
–	TcrPDEC-S	TcrPDEC	–	36 S	Kunz (2005)

(continued)

Table 1 (continued)

Enzyme family	–	Older designations	Protein and gene identification	Number	References
–	TcrPDEC-S ^d	TcPDE4	Q53160 Tc00.1047053506697.20 Q4ZHU6 n.a.	n.a.	Alonso et al. (2006)
–	LmjPDEC	–	Q4FXD4 LmjF29.2680	29	–
–	LinPDEC	–	A4IaX6 LinJ29.2990	29	–
–	LbrPDEC	–	A4HHS5 LbrM29.2730	29	–
–	–	–	–	–	–
PDED	TbrPDED	–	Q57V52 Tb927.3.3340	3	–
–	TcrPDED-P	–	Q4E664 Tc00.1047053508153.260	36 P	–
–	TcrPDED-S	–	Q4D9B7 Tc00.1047053510323.50	36 S	–
–	LmjPDED	–	Q4FXF8 LmjF29.2440	29	–
–	LinPDED	–	A4I4V8 LinJ29.2800	29	–
–	LbrPDED	–	A4HHP3 LbrM29.2460	29	–

Column 5: number of the chromosome on which the gene is located

Tbr: *Trypanosoma brucei*, Tcr: *Trypanosoma cruzi*, Lmj: *Leishmania major*, Lin: *L. infantum*, Lbr: *L. braziliensis*, EMBL: UniProtKB/TrEMBL accession number, TriTryp: TriTryp database gene nomenclature; n.a.: data not available

^aGenomic locus in Trytryp database incorrectly assembled

^bGAF-A domain exchanged with the GAF-A domain of TbrPDEB1 in all strains derived from Lister 427

^cPseudogene with internal stop codon

^dPossibly an allelic variant of Q53160

PDEAs of all three organisms is that they can complement PDE-deficient yeast strains, indicating that they are active in the yeast cell, but that no biochemically measurable enzyme activity could be detected in yeast cell lysates. This is in surprising contrast to findings with the other PDEs (see below).

PDEB. In all kinetoplastids, two isoforms of this enzyme are coded for by two tandemly arranged genes, PDEB1 and PDEB2. These most likely represent an ancient gene duplication that predates the divergence of the kinetoplastids. In *T. brucei*, both genes are expressed at similar levels in both bloodstream and procyclic forms. The respective polypeptides consist of 930 (TbrPDEB1) and 925 (TbrPDEB2) amino acids and contain two GAF domains in the N-terminal region (D₂₃₄–E₅₅₄ in TbrPDEB1), followed by a PDE catalytic domain (V₅₈₆–R₉₀₈ in TbrPDEB1). The GAF and catalytic domains are highly conserved between TbrPDEB1 and TbrPDEB2 (96.3 and 88.3% amino acid identity, respectively). In an ancestor of a series of a popular laboratory strain of *T. brucei* (the Lister 427

derivatives), the TbrPDEB1 and TbrPDEB2 genes have undergone a partial gene conversion, exchanging very precisely the GAF-A domain of TbrPDEB2 with the corresponding domain of the upstream gene TbrPDEB1. Thus, all these strains are heterozygous for TbrPDEB2, carrying one allele of TbrPDEB2a (the wild-type version) and one allele of TbrPDEB2b (with a GAF-A domain that originates from TbrPDEB1). While this gene conversion has no functional consequences, it provides a convenient genetic tool for unambiguous identification of trypanosome strains (Kunz et al. 2009a).

Both enzymes are cAMP-specific, with K_m values in the order of 5 μM (Zoraghi and Seebeck 2002; Rascon et al. 2002; Seebeck et al. unpublished data). The GAF-A domains of both enzymes bind cAMP and cGMP with high affinity (K_D cAMP 16 nM; K_D cGMP 275 nM; Laxman et al. 2005; Seebeck et al. unpublished data), suggesting that the domains might function as highly sensitive cyclic nucleotide sensors. Between the two GAF domains is a predicted PKA phosphorylation site (K₃₈₇–S₃₉₀). This site is conserved in all kinetoplastid PDEB1 and PDEB2 enzymes and may be functional. At the C-terminus of TbrPDEB1, residues S₉₂₀, S₉₂₂, and S₉₂₃ have been identified in the *T. brucei* phosphoproteome, while none of the corresponding residues of TbrPDEB2 are represented (Nett et al. 2009).

The two PDEs have also been investigated in *T. cruzi* [TcrPDEB1 (Diaz-Benjumea et al. 2006) and TcrPDEB2 (D'Angelo et al. 2004)] and shown to have very similar characteristics. The mRNAs for both enzymes are present in all three developmental stages (amastigotes, trypomastigotes, and epimastigotes). Both enzymes are cAMP-specific, with K_m values of 2.8 μM (TcrPDEB1 (Diaz-Benjumea et al. 2006) and 7.8 μM (TcrPDEB2 (D'Angelo et al. 2004)). Interestingly, the fluorescent cAMP derivative MANT-cAMP is hydrolyzed with a K_m very similar to that of cAMP [1.8 vs. 2.8 μM (Diaz-Benjumea et al. 2006)]. An analysis of cyclic nucleotide binding to the GAF-A domains of the *T. cruzi* enzymes revealed significant differences between the *T. brucei* homologues: cAMP bound to the GAF-A domain of TcrPDEB1 with a K_D of 970 nM and the corresponding values for TcrPDEB2 were 190 nM (by filter binding) or 520 nM (by isothermal microcalorimetry). The GAF-A domain of TcrPDEB1 did not measurably bind cGMP, while the GAF-A domain of TcrPDEB2 bound cGMP with a K_D of 2.5 mM (Diaz-Benjumea et al. 2006). Further experimentation is required to resolve whether these low binding affinities of the *T. cruzi* GAF-A domain are in fact genuine.

PDEC. All kinetoplastid genomes contain one copy of PDEC. The predicted proteins (about 900 amino acids) are unusual in that they contain a FYVE-related domain (Kutateladze 2007) at the N-terminus, followed by a coiled-coil region. This architecture suggests that the enzymes might be located in the endosomal compartment. The catalytic domain is situated approximately in the middle of the polypeptide and is followed by a rather long C-terminal region (298 amino acids in TbrPDEC).

TcrPDEC from *T. cruzi* was independently characterized by two groups (Kunz et al. 2005; Alonso et al. 2006), who obtained different values for its specificity. While Alonso et al. (2006) reported that the enzyme is cAMP-specific (K_m 20 μM), Kunz et al. (2005) found that it hydrolyzes both cAMP (K_m 30 μM) and cGMP (K_m 78 μM) with similar V_{max} . The reason for this discrepancy remains unresolved.

TcrPDEC is weakly inhibited by etazolate, trequinsin, and dipyridamole (IC_{50} values in the 1 μ M range), while it is highly resistant to many other PDE inhibitors, including rolipram, cilostamide, zardaverin, IBMX, and papaverine.

PDED. All kinetoplastid genomes contain one copy of this putative PDE. The DNA sequence predicts a polypeptide of about 670 amino acids length containing a class 1 catalytic domain at the C-terminus. Currently, only the sequences are known and no experimental data are available in any species.

2.2 Biological Function

PDEA. Homozygous deletion of TbrPDEA has only minor effects on cell growth in culture. A slight increase in the steady-state cAMP concentration is observed in the deletion mutant, and generation time is prolonged by about 20%. However, the deletion mutants remain fully infectious for tsetse flies (Gong et al. 2001). The currently available information suggests that TbrPDEA is a minor activity in both bloodstream and procyclic trypanosomes and that it is not essential for cell viability. Its biological role might rather consist of the modulation of cAMP signals, analogous to what has been suggested for the high- K_m class 2 PDE of *S. cerevisiae* (Ma et al. 1999).

PDEB. In striking contrast, TbrPDEB1 and TbrPDEB2 are essential enzymes in *T. brucei*. The lack of success in early attempts to knock out these genes in bloodstream form *T. brucei* indicated that they might be required for cell survival (Zoraghi and Seebeck 2002). The development of a genetically encoded inducible RNA interference system in *T. brucei* enabled reexamination of the biological role of the two enzymes; RNAi against either of the two enzymes does not produce an observable phenotype. However, simultaneous RNAi against both PDEs leads to complete cell lysis within 48 h after induction of RNAi (Oberholzer et al. 2007). Simultaneous RNAi, but not RNAi against the individual enzymes, results in a dramatic increase (100-fold) in steady-state intracellular cAMP concentration within a few hours of inducing RNAi. RNAi against TbrPDEB1 and TbrPDEB2 does not increase the mRNA levels of the other three PDEs, TbrPDEA, TbrPDEC, or TbrPDED, indicating that the expression of these PDEs is not subject to control by cAMP.

Further experiments demonstrated that TbrPDEB1 and TbrPDEB2 can functionally complement each other, despite the fact that their subcellular localization is clearly different (Oberholzer et al. 2007; Kunz et al. 2009a). This finding is somewhat at variance with current concepts of neatly localized cAMP microdomains and precisely anchored members of the signaling cascade. Rather, it suggests that maintenance of the overall steady-state concentration of cAMP by a PDE located anywhere in the cell is sufficient for preserving many of the basic functions of the cell. This conclusion is also supported by the complementation of PDE-deficient yeast cells by various heterologous PDEs (Kunz et al. 2004; Johner et al. 2006;

Alonso et al. 2007; Ivey et al. 2008), which allows the yeast cell to become stress-resistant without the requirement for precise subcellular localization.

The genetically encoded, inducible RNA interference system (see above) also enabled *in vivo* infection experiments. When animals were infected with the transgenic trypanosomes, the strain was fully infective and the animals reached a parasitemia of $>1 \times 10^8$ trypanosomes/mL blood within 3 days. However, when the animals received doxycycline (a tetracycline analogue) in the drinking water, starting 2 days before and continuing throughout the entire course of infection, no trypanosomes were observed at any time. In other experiments designed to determine whether the ablation of PDE activity was sufficient to interrupt an ongoing infection, animals were infected in the absence of doxycycline. The infection was allowed to take its course until the parasitemia reached approximately 1×10^7 /mL. When doxycycline was added to the drinking water at this point, the infection was cleared within 24 h (Oberholzer et al. 2007). Parasite clearance was independent of the host immune system, since oral doxycycline also completely eliminated the trypanosomes in immunosuppressed animals.

3 The Promise and the Challenge of Targeting PDEs for Treatment of the Kinetoplastid Diseases

3.1 Therapeutic Potential of Kinetoplastid PDE Inhibitors

The extensive experiments with RNAi described above unambiguously validate PDEB1/B2 as essential proteins *in vitro* and *in vivo*. Their ablation by RNAi creates a runaway accumulation of intracellular cAMP that leads to cell cycle defects and ultimately to cell death. The reasonable inference has been made that inhibition of the kinetoplastid PDEBs should similarly lead to the clearance of parasite populations. On the one hand, the failure of classical PDE inhibitors to achieve this has left those trying to develop such inhibitors without positive controls; on the other hand, this clearly shows that the binding pockets are sufficiently different for selective inhibitors to be possible. Apart from the dearth of positive controls, a more serious challenge is that both PDEB1 and PDEB2 must be strongly inhibited to achieve a significant change in cAMP levels and consequently in cell survival (Oberholzer et al. 2007).

A series of compounds developed at Altana Pharma and Nycomed were found to inhibit both enzymes in the nanomolar range and displayed antitrypanosomal activity at concentrations similar to its TbrPDEB IC_{50} (H. Tenor, G.J. Sterk, and T. Seebeck, unpublished). Using the standard assay based on the fluorophore Alamar blue (resazurin) (Gould et al. 2008), the lead compound, BYK54826, displayed a trypanocidal EC_{50} value of 80 ± 10 nM. It also rapidly and dose-dependently increased levels of cAMP in bloodstream *T. b. brucei*. As little as 100 nM of this compound elevated the cAMP level by threefold in 10 min (Fig. 3);

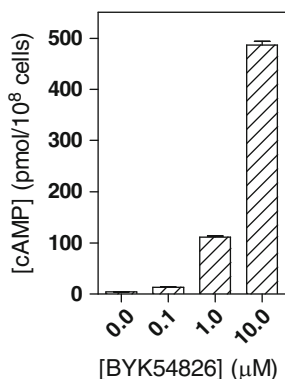


Fig. 3 Effect of BYK54826 on cAMP levels in *T. b. brucei*. Trypanosomes (2.5×10^6 Lister 427 bloodstream forms) were incubated for 10 min in the presence or absence of BYK54826 at the concentrations indicated. Incubations were stopped by 30 s centrifugation at $13,000 \times g$ followed by the addition of 150 μl ice-cold 0.1 M HCl to the cell pellet; cAMP was determined using the Direct Cyclic AMP ELISA kit (Assay Designs) according to the manufacturer's instructions

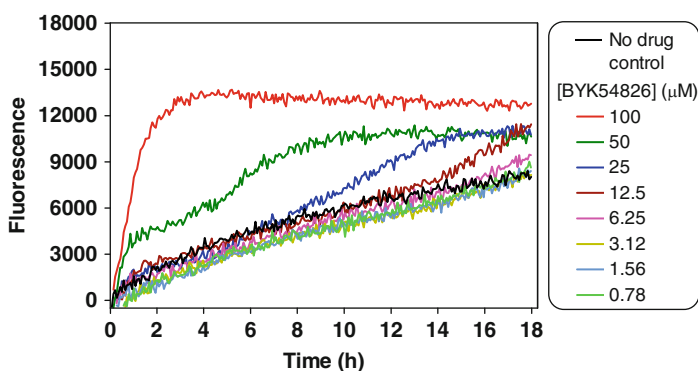


Fig. 4 Effect of BYK54826 on plasma membrane permeability in trypanosomes. Bloodstream form trypanosomes (strain 427, 5×10^6 cells/mL in HMI-9 medium plus 10% FCS) were incubated at 37°C under 5% CO_2 for 18 h in a FLUOstar OPTIMA fluorimeter in the presence of 6 $\mu\text{g}/\text{mL}$ propidium iodide (PI). Survival was monitored by measuring fluorescence of PI, which enters live trypanosomes only extremely slowly and fluoresces after binding to nucleic acids (Gould et al. 2008). All traces were recorded simultaneously in a white-bottomed 96-well plate

0.3 μM caused a 20-fold rise within 20 min and remaining elevated at this level for hours (not shown); 1 μM increased cAMP levels up to 50-fold, similar to the observations with PDEB RNAi (Oberholzer et al. 2007). In contrast, dipyrindamole (40 μM) and etazolol (100 μM), both low-affinity inhibitors of TbrPDEBs (Zoraghi and Seebeck 2002), did not affect trypanosomal cAMP levels. However, the rapid cAMP response did not affect cellular survival for hours. As shown in Fig. 4, only extreme concentrations of BYK54286 of 50 or 100 μM had a rapid effect on parasite viability. Lower concentrations had no measurable effect on this parameter for up to

8 h (25 μM), 14 h (12.5 μM), or even longer – despite concentrations of the test compound being more than two orders of magnitude above the EC_{50} value.

Although BYK54826 had only a slow effect on trypanosome viability, it appeared to rapidly halt cell division and growth, indicating that PDE inhibition leads to cell cycle aberrations. Figure 5 shows that, after 8 h of incubation with 1 μM BYK54826, trypanosomes accumulate in the G2/M phase of the cell cycle, with a greatly increased proportion of cells containing double the normal amount of DNA (i.e., two nuclei). Longer incubations demonstrate the appearance of a further peak, representing four nuclei (not shown).

The cell cycle of trypanosomes is relatively complex, with kinetoplasts dividing first, before the nuclei, in a process that is linked to the generation of the new flagellum (McKean 2003). Fluorescence microscopy after DAPI staining showed a statistically significant ($P < 0.001$) increase in trypanosomes with more than two nuclei and kinetoplasts after 12 h of incubation with 1 μM BYK54826. It thus appears that the inhibition of TbrPDEB1/B2 does not stop DNA synthesis or indeed the separation into new kinetoplasts and nuclei, but does prevent the completion of cytokinesis into independent daughter cells, leading to the formation of nonviable, multinucleated cells. Elevation of cAMP levels does not disrupt the coordination between the kinetoplast and nuclear division cycles: we saw no evidence of cells with more nuclei than kinetoplasts, for instance, and division of kinetoplasts continued to precede nuclear division. Cells that reached the four nuclei stage disintegrated within a few hours, leading to the clearance of the parasite population.

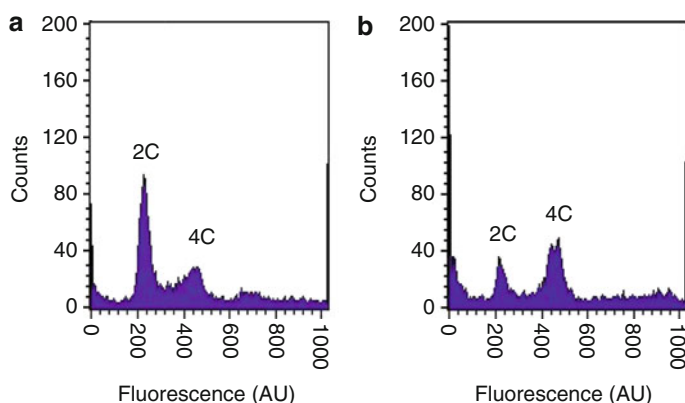


Fig. 5 BYK54826 causes cell cycle arrest in *T. brucei*. Flow cytometric analysis of the DNA content of bloodstream forms of strain 427 trypanosomes, (a) untreated and (b) treated with 1 μM BYK54826, incubated under normal culturing conditions for 8 h. The histograms show the number of cells with a particular fluorescence intensity that correlates with the amount of DNA in the cell. The peaks labeled 2C represent the number of cells in the sample with a complete diploid complement of DNA, having two copies of the genome; the peaks labeled 4C represent the cells that have replicated their DNA but not yet undergone cytokinesis, therefore having four copies of the genome

These results are entirely consistent with the earlier observations after TbrPDEB knockdown with tetracycline-inducible RNAi (Oberholzer et al. 2007).

These results provide pharmacological validation of the B class of kinetoplastid phosphodiesterases as promising drug targets.

3.2 Crystal Structure of *Leishmania major* Phosphodiesterase B1 and Implications for Selectivity of Parasite PDE Inhibitors

3.2.1 Structural Similarity and Variation Between LmjPDEB1 and Human PDEs

The structure of the catalytic domain of LmjPDEB1 (residues 582–940) in complex with IBMX is the first and only structure of a parasite PDE determined to date (Wang et al. 2007). The catalytic domain contains 2 divalent metal ions and 16 α -helices folded in an overall architecture similar to that of human PDEs (Wang et al. 2007; Ke and Wang 2007). The superposition revealed four regions that exhibit significant differences between the structures of LmjPDEB1 and human PDEs (Fig. 6). The most significant differences are associated with the H- and M-loops, which have positional shifts of as much as 3 Å for their C α atoms, about twice the overall root-mean-squared deviation between the structures of LmjPDEB1 and human PDEs. Since the H- and M-loops are directly involved in interaction with inhibitors (Huai et al. 2004; Wang et al. 2006; Ke and Wang 2007), their significant positional shifts most likely have pharmacological implications, but further characterization is needed to understand their exact roles.

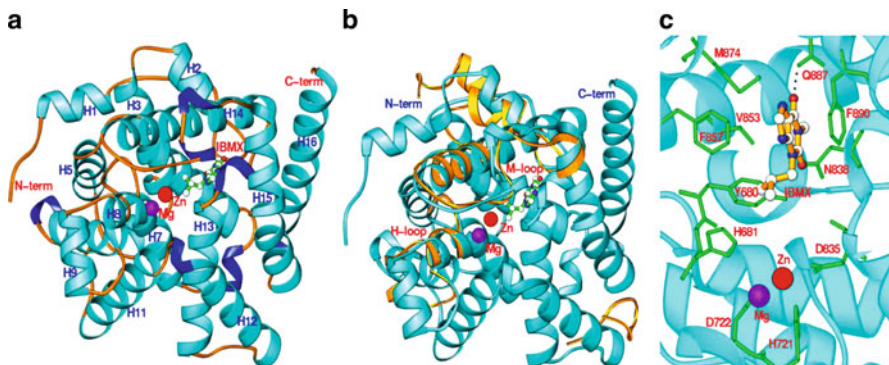


Fig. 6 The structure of LmjPDEB1 (Wang et al. 2007). (a) *Ribbon diagram* of the catalytic domain of LmjPDEB1 in complex with the nonselective inhibitor, IBMX. (b) Comparison of LmjPDEB1 (cyan ribbons) with PDE4D2. Only PDE4D loops (gold ribbons) with large positional differences are shown. (c) IBMX binding at the active site of LmjPDEB1. The dotted line represents the hydrogen bond between IBMX and the side chain of glutamine

3.2.2 IBMX Binding

IBMX is a nonselective inhibitor of most human PDE families, but it only weakly inhibits LmjPDEB1, with an IC_{50} of 580 μ M. IBMX binds to the active site of LmjPDEB1: the O6 atom of the xanthine ring of IBMX forms a hydrogen bond with Ne2 of Gln887 in LmjPDEB1 (Fig. 6). The xanthine ring stacks against Phe890 of LmjPDEB1 and also forms van der Waals' contacts with residues Tyr680, Asn838, Val853, and Phe857. This pattern of IBMX binding is similar to that found in human PDE-IBMX complexes (Ke and Wang 2007). In fact, the hydrogen bond with the invariant glutamine and stacking against phenylalanine are two characteristics for binding of not only IBMX in LmjPDEB1, but also almost all inhibitors of human PDEs (Ke and Wang 2007), whether or not they are selective. Notably, the xanthine ring of IBMX in the LmjPDEB1 structure has the same orientation as that in PDE3B, PDE5A, and PDE9A, but is rotated by about 180° in PDE4D, PDE7A, and PDE8A (Wang et al. 2008). The different orientations of IBMX in the PDE structures may just mean that the active site pockets of PDEs, which are much larger than the volume of most PDE inhibitors, can accommodate multiple inhibitor conformations.

3.2.3 Subtle but Significant Differences Between the Active Sites of LmjPDEB1 and Human PDEs

A structural comparison shows an overall similarity between the active sites of LmjPDEB1 and human PDEs, including the four metal-binding residues (His685, His721, Asp722, and Asp835), the key residues such as His681 and His725 for the catalysis, and Phe890 for the inhibitor binding. However, Gln887 of LmjPDEB1 shows a significant positional displacement. Since the invariant glutamine (Gln887 of LmjPDEB1) donates hydrogen bonds and plays an essential role in substrate and inhibitor binding (Ke and Wang 2007), the positional variation of Gln887 must be an important factor that diminishes the binding of the human PDE inhibitors to LmjPDEB1. In addition, alterations in Asn838, Val839, Ser846, Met874, and Gly886 of LmjPDEB1 are also significant and may thus affect inhibitor binding. In particular, Met874 and Gly886 appear to serve as two gating residues controlling access to a unique pocket that may be valuable for the design of LmjPDE-selective inhibitors.

3.2.4 A Unique Pocket of LmjPDEB1 for Inhibitor Binding

A unique pocket called the L-pocket has been identified in LmjPDEB1 (Wang et al. 2007). It lies adjacent to the main inhibitor binding pocket and next to the cyclic pentanyl ring of rolipram in the PDE4 structure (Fig. 7). This pocket is made up of residues from the M-loop and helix H14, including Thr854, Tyr858, Met874, Asn881, Leu883, and Gly886 in LmjPDEB1. Thr854 and Tyr858 of helix H14 and Leu883 and Gly886 of helix H15 form two walls of the pocket, while the fragment of

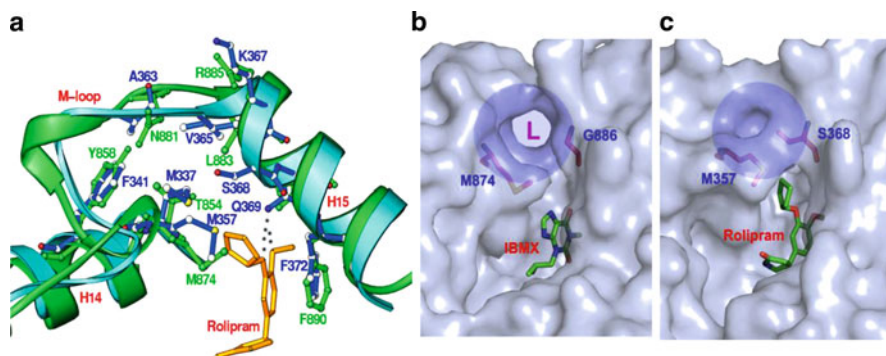


Fig. 7 Putative *Leishmania* parasite pocket (L-pocket; Wang et al. 2007). (a) Superposition of LmjPDEB1 (green ribbons, sticks, and labels) over PDE4D2 (cyan ribbons, blue sticks, and labels). (b) Surface presentation of the unique L-pocket of LmjPDEB1 (shaded circle labeled with L) and the equivalent region in PDE4D (c)

the M-loop around Asn881 constitutes the third side of the pocket (Fig. 7a). Interestingly, the bottom of the pocket is open to the molecular surface of LmjPDEB1. Thus, the pocket looks more like an open channel. It has a size capable of accommodating a five-membered ring and shows mixed hydrophilic and hydrophobic characteristics. At the entrance to the pocket, the two residues Met874 and Gly886 of LmjPDEB1 are separated by 7.5 Å between their C α atoms and serve as a gate open to the LmjPDEB1 L-pocket.

In human PDE families, however, the amino acids corresponding to the two entry residues are sitting so close to each other as to isolate the L-pockets from the main inhibitor binding sites. For example, Met847 and Leu858 in PDE2A, Phe976 and Leu987 in PDE3B, Met357 and Ser368 in PDE4D2, and Leu401 and Ile412 in PDE7A all block the entry to the L-pocket. In PDE9A, Phe441 and Ala452 do not block the path, but Val447 moves over by 3 Å into the L-pocket and thus fills it up. In PDE10A, Met713 and Gly725 are separated by 6.7 Å, providing an access route to the L-pocket, but this is blocked by Tyr693. It is interesting to note that a gating glycine (Gly886 in LmjPDEB1) exists in four of the five LmjPDE families, but only in human PDE10, and has the largest positional difference among the active site residues. Since glycine is unique in that its backbone conformation can freely assume any angle, this residue must represent a fundamental difference between most human and *Leishmania* PDEs. In short, we believe that the L-pocket is useful for the development of *Leishmania*-selective inhibitors.

3.3 Challenges

T. brucei, *T. cruzi*, and *Leishmania* are closely related parasites, causing very different diseases, occupy different environments in the human hosts, and hence the development of drugs for each indication involves both common and disease-specific

challenges. A fundamental issue relevant to all three genera involves the identification of selective inhibitors that target the essential PDEs. Ideally, cross-reactivity with the various human PDE families should be minimal. The feasibility of finding a solution to this challenge can be judged by comparison with the successful development of selective inhibitors for the human PDEs. The identification of drugs specific for human PDE3, PDE4, and PDE5 illustrates that genetic differences between PDE families can be sufficient to enable the development of selective inhibitors (Bender and Beavo 2006). Bearing in mind the urgent need for new therapies and the desirability of a relatively short duration of treatment, the requirement for very stringent selectivity might be relaxed somewhat if the drug was efficacious and its side effects were acceptable and reduced compared to current treatments.

Another important consideration is minimization of the development of drug resistance. This has been best studied in *T. brucei*, where mutations in the P2 purine transporter, TbAT1, and the high-affinity pentamidine transporter, HAPT1, result in decreased importation of melarsoprol and pentamidine into the cell, reducing their trypanocidal efficacy (De Koning 2008). Melarsoprol resistance has also been demonstrated in laboratory strains overexpressing the multidrug resistance-associated protein, TbMRPA, which showed increased export of drugs from the cell (Luscher et al. 2006). A preliminary assessment of candidate drug interactions with these transporters, especially PDE inhibitors with purine-like scaffolds, may help circumvent these common resistance mechanisms. Resistance is less likely to occur with compounds that enter the cell by more than one route, e.g., a combination of active and passive transport or via multiple active transport mechanisms (transporter- or receptor-mediated). Lack of interaction with TbAT1, HAPT1, and TbMRPA would also avoid problems of cross-resistance.

One of the main disease-specific challenges that must be met in order for a PDE inhibitor to progress from a hit to lead compound is its ability to gain access to the parasite within the host. In the case of *T. brucei*, it is essential that the compound be able to cross the blood–brain barrier for it to be effective against late-stage CNS disease. For *T. cruzi* and *Leishmania* which, unlike *T. brucei*, are intracellular parasites, the compound must not only be able to cross the host cell membrane, but also the parasite membrane in sufficiently high concentrations, and at a sufficient rate, to kill the parasite.

Additionally, any potential therapy for neglected diseases must take into consideration the inability of the patient to pay for treatment and the lack of adequate hospital facilities. Ideally, compounds should be cheap to produce, stable in the environments where they will be used, have a short treatment course, and be easy to administer (preferably oral) to ensure patient compliance and to diminish the risk of resistance.

4 Perspective

Although past progress has been slow, more recently, significant strides have been made in our understanding of the importance of cAMP signaling in kinetoplastid function. These findings provide genetic and pharmacological validation of the

PDEs as novel drug targets for diseases caused by the kinetoplastid parasites. In the bloodstream form of *T. brucei*, this has been demonstrated by the observation that normal flagellar function, completion of cytokinesis, and ultimately cell survival are all absolutely dependent on PDEs. The lethal phenotype observed following PDE knockdown could be due to two possibilities: the absence of the protein, leading to disruption of an associated signaling complex, or the loss of enzymatic function. The finding that pharmacological inhibition of PDE activity in *T. brucei* mimics the lethal phenotype resulting from PDE knockdown supports the latter.

While selective inhibitors of kinetoplastid PDEs have yet to be identified, the first kinetoplastid PDE crystal structure has been elucidated and shows differences from mammalian PDEs that could be exploited in the design of selective compounds. The similarity between TbrPDEB, LmjPDEB, and TcrPDEB isoforms raises the possibility of a single inhibitor for all three PDE isoforms and the potential for one drug for several diseases. Whether this is ultimately feasible will depend on many additional factors, including those outlined in Sect. 3.3. The development of compounds with promising preclinical profiles can realistically be pursued in partnership with one of the several not-for-profit organizations that have been specifically created to meet the need for new drugs against neglected diseases (Cohen 2006; Butler 2007). Further studies will be required to determine whether the possibilities suggested by this preliminary evidence can be realized for the development of PDE inhibitors as a new class of drug to combat parasitic diseases.

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References

- Alonso GD, Schoijet AC, Torres HN, Flawia MM (2006) TcPDE4, a novel membrane-associated cAMP-specific phosphodiesterase from *Trypanosoma cruzi*. *Mol Biochem Parasitol* 145:40–49
- Alonso GD, Schoijet AC, Torres HN, Flawia MM (2007) TcrPDEA1, a cAMP-specific phosphodiesterase with atypical pharmacological properties from *Trypanosoma cruzi*. *Mol Biochem Parasitol* 152:72–79
- Alvar J, Aparicio P, Aseffa A, Den BM, Canavate C, Dedet JP, Gradoni L, Ter HR, Lopez-Velez R, Moreno J (2008) The relationship between leishmaniasis and AIDS: the second 10 years. *Clin Microbiol Rev* 21:334–359, Table
- Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, Krishna S (2003) The trypanosomiases. *Lancet* 362:1469–1480
- Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 58:488–520
- Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, Lennard NJ, Caler E, Hamlin NE, Haas B, Bohme U, Hannick L, Aslett MA, Shallom J, Marcello L, Hou L, Wickstead B, Almsmark UC, Arrowsmith C, Atkin RJ, Barron AJ, Bringaud F, Brooks K, Carrington M, Cherevach I, Chillingworth TJ, Churcher C, Clark LN, Corton CH, Cronin A, Davies RM, Doggett J, Djikeng A, Feldblyum T, Field MC, Fraser A, Goodhead I, Hance Z, Harper D, Harris BR, Hauser H, Hostetler J, Ivans A, Jagels K, Johnson D, Johnson J, Jones K, Kerhornou AX, Koo H, Lark N, Landfear S, Larkin C, Leech V, Line A, Lord A, Macleod A, Mooney PJ, Moule S, Martin DM, Morgan GW, Mungall K, Norbertczak H, Ormond D, Pai G,

- Peacock CS, Peterson J, Quail MA, Rabbinowitsch E, Rajandream MA, Reitter C, Salzberg SL, Sanders M, Schobel S, Sharp S, Simmonds M, Simpson AJ, Tallon L, Turner CM, Tait A, Tivey AR, Van AS, Walker D, Wanless D, Wang S, White B, White O, Whitehead S, Woodward J, Wortman J, Adams MD, Embley TM, Gull K, Ullu E, Barry JD, Fairlamb AH, Opperdoes F, Barrell BG, Donelson JE, Hall N, Fraser CM, Melville SE, El-Sayed NM (2005) The genome of the African trypanosome *Trypanosoma brucei*. *Science* 309:416–422
- Black S, Seed J (2001) World class parasites: volume 1 the African trypanosomes. Kluwer Academic Publishers, Boston, MA
- Breidbach T, Ngazoa E, Steverding D (2002) *Trypanosoma brucei*: in vitro slender-to-stumpy differentiation of culture-adapted, monomorphic bloodstream forms. *Exp Parasitol* 101: 223–230
- Broadhead R, Dawe HR, Farr H, Griffiths S, Hart SR, Portman N, Shaw MK, Ginger ML, Gaskell SJ, McKean PG, Gull K (2006) Flagellar motility is required for the viability of the bloodstream trypanosome. *Nature* 440:224–227
- Brun R, Blum J, Chappuis F, Burri C (2010) Human African trypanosomiasis. *Lancet* 375(9709): 148–159
- Butler D (2007) Lost in translation. *Nature* 449:158–159
- Castro JA, de Mecca MM, Bartel LC (2006) Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis). *Hum Exp Toxicol* 25:471–479
- Chirac P, Torreele E (2006) Global framework on essential health R&D. *Lancet* 367:1560–1561
- Cohen J (2006) Global health. Public-private partnerships proliferate. *Science* 311:167
- Croft SL, Coombs GH (2003) Leishmaniasis—current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol* 19:502–508
- D'Angelo MA, Sanguineti S, Reece JM, Birnbaumer L, Torres HN, Flawia MM (2004) Identification, characterization and subcellular localization of TcPDE1, a novel cAMP-specific phosphodiesterase from *Trypanosoma cruzi*. *Biochem J* 378:63–72
- De Koning HP (2008) Ever-increasing complexities of diamidine and arsenical crossresistance in African trypanosomes. *Trends Parasitol* 24:345–349
- Delespaux V, De Koning HP (2007) Drugs and drug resistance in African trypanosomiasis. *Drug Resist Updat* 10:30–50
- Diaz-Benjumea R, Laxman S, Hinds TR, Beavo JA, Rascon A (2006) Characterization of a novel cAMP-binding, cAMP-specific cyclic nucleotide phosphodiesterase (TcrPDEB1) from *Trypanosoma cruzi*. *Biochem J* 399:305–314
- Farrell J (2002) World class parasites: volume 4 Leishmania. Kluwer Academic Publishers, Boston
- Gong KW, Kunz S, Zoraghi R, Kunz RC, Brun R, Seebeck T (2001) cAMP-specific phosphodiesterase TbPDE1 is not essential in *Trypanosoma brucei* in culture or during midgut infection of tsetse flies. *Mol Biochem Parasitol* 116:229–232
- Gould MK, Vu XL, Seebeck T, De Koning HP (2008) Propidium iodide-based methods for monitoring drug action in the kinetoplastidae: comparison with the Alamar Blue assay. *Anal Biochem* 382:87–93
- Huai Q, Liu Y, Francis SH, Corbin JD, Ke H (2004) Crystal structures of phosphodiesterases 4 and 5 in complex with inhibitor 3-isobutyl-1-methylxanthine suggest a conformation determinant of inhibitor selectivity. *J Biol Chem* 279:13095–13101
- Huang H, Werner C, Weiss LM, Wittner M, Orr GA (2002) Molecular cloning and expression of the catalytic subunit of protein kinase A from *Trypanosoma cruzi*. *Int J Parasitol* 32:1107–1115
- Huang H, Weiss LM, Nagajyothi F, Tanowitz HB, Wittner M, Orr GA, Bao Y (2006) Molecular cloning and characterization of the protein kinase A regulatory subunit of *Trypanosoma cruzi*. *Mol Biochem Parasitol* 149:242–245
- Hunger-Glaser I, Seebeck T (1997) Deletion of the genes for the paraflagellar rod protein PFR-A in *Trypanosoma brucei* is probably lethal. *Mol Biochem Parasitol* 90:347–351
- Ivey FD, Wang L, Demirbas D, Allain C, Hoffman CS (2008) Development of a fission yeast-based high-throughput screen to identify chemical regulators of cAMP phosphodiesterases. *J Biomol Screen* 13:62–71

- Johner A, Kunz S, Linder M, Shakur Y, Seebeck T (2006) Cyclic nucleotide specific phosphodiesterases of *Leishmania major*. *BMC Microbiol* 6:25
- Ke H, Wang H (2007) Crystal structures of phosphodiesterases and implications on substrate specificity and inhibitor selectivity. *Curr Top Med Chem* 7:391–403
- Kunz S, Kloeckner T, Essen LO, Seebeck T, Boshart M (2004) TbpPDE1, a novel class I phosphodiesterase of *Trypanosoma brucei*. *Eur J Biochem* 271:637–647
- Kunz S, Oberholzer M, Seebeck T (2005) A FYVE-containing unusual cyclic nucleotide phosphodiesterase from *Trypanosoma cruzi*. *FEBS J* 272:6412–6422
- Kunz S, Beavo JA, D'Angelo MA, Flawia MM, Francis SH, Johner A, Laxman S, Oberholzer M, Rascon A, Shakur Y, Wentzinger L, Zoraghi R, Seebeck T (2006) Cyclic nucleotide specific phosphodiesterases of the kinetoplastida: a unified nomenclature. *Mol Biochem Parasitol* 145:133–135
- Kunz S, Luginbuehl E, Seebeck T (2009a) Gene conversion transfers the GAF-A domain of phosphodiesterase TbrPDEB1 to one allele of TbrPDEB2 of *Trypanosoma brucei*. *PLoS Negl Trop Dis* 3:e455
- Kunz S, Minca M, Luginbuehl E, Bregy P, Seebeck T (2009b) Cyclic nucleotide signaling in the kinetoplastids. *Handbook of cell signaling*. Academic Press, New York, NY, pp 1543–1547
- Kutateladze TG (2007) Mechanistic similarities in docking of the FYVE and PX domains to phosphatidylinositol 3-phosphate containing membranes. *Prog Lipid Res* 46:315–327
- Laxman S, Beavo JA (2007) Cyclic nucleotide signaling mechanisms in trypanosomes: Possible targets for therapeutic agents. *Molecular Interventions* 7:203–215
- Laxman S, Rascon A, Beavo JA (2005) Trypanosome cyclic nucleotide phosphodiesterase 2B binds cAMP through its GAF-A domain. *J Biol Chem* 280:3771–3779
- Laxman S, Riechers A, Sadilek M, Schwede F, Beavo JA (2006) Hydrolysis products of cAMP analogs cause transformation of *Trypanosoma brucei* from slender to stumpy-like forms. *Proc Natl Acad Sci USA* 103:19194–19199
- Luscher A, Nerima B, Maser P (2006) Combined contribution of TbAT1 and TbMRPA to drug resistance in *Trypanosoma brucei*. *Mol Biochem Parasitol* 150:364–366
- Ma P, Wera S, van DP, Thevelein JM (1999) The PDE1-encoded low-affinity phosphodiesterase in the yeast *Saccharomyces cerevisiae* has a specific function in controlling agonist-induced cAMP signaling. *Mol Biol Cell* 10:91–104
- Mancini PE, Patton CL (1981) Cyclic 3', 5'-adenosine monophosphate levels during the developmental cycle of *Trypanosoma brucei brucei* in the rat. *Mol Biochem Parasitol* 3:19–31
- McKean PG (2003) Coordination of cell cycle and cytokinesis in *Trypanosoma brucei*. *Curr Opin Microbiol* 6:600–607
- Nett IR, Martin DM, Miranda-Saavedra D, Lamont D, Barber JD, Mehlert A, Ferguson MA (2009) The phosphoproteome of bloodstream form *Trypanosoma brucei*, causative agent of African sleeping sickness. *Mol Cell Proteomics* 8:1527–1538
- Oberholzer M, Marti G, Baresic M, Kunz S, Hemphill A, Seebeck T (2007) The *Trypanosoma brucei* cAMP phosphodiesterases TbrPDEB1 and TbrPDEB2: flagellar enzymes that are essential for parasite virulence. *FASEB J* 21:720–731
- Olliaro PL, Guerin PJ, Gerstl S, Haaskjold AA, Rottingen JA, Sundar S (2005) Treatment options for visceral leishmaniasis: a systematic review of clinical studies done in India, 1980–2004. *Lancet Infect Dis* 5:763–774
- Paine M, Wang M, Boykin D, Wilson W, De Koning H, Olson C, Polig G, Burri C, Brun R, Murilla G, Thuita JK, Barrett M, Tidwell R (2010) Diamidines for human African trypanosomiasis. *Curr Opin Invest Drugs* In Press:
- Priotto G, Kasparian S, Mutombo W, Nguouama D, Ghorashian S, Arnold U, Ghabri S, Baudin E, Buard V, Kazadi-Kyanza S, Ilunga M, Mutangala W, Pohlig G, Schmid C, Karunakara U, Torrelee E, Kande V (2009) Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *Lancet* 374:56–64

- Rangel-Aldao R, Triana F, Comach G, Abate T, Fernandez V, Mahon-Pratt D (1988) Intracellular signaling transduction in the differentiation of *Trypanosoma cruzi*: role of cAMP. *Arch Biol Med Exp (Santiago)* 21:403–408
- Rascon A, Soderling SH, Schaefer JB, Beavo JA (2002) Cloning and characterization of a cAMP-specific phosphodiesterase (TbPDE2B) from *Trypanosoma brucei*. *Proc Natl Acad Sci USA* 99:4714–4719
- Reed SL, Fierer AS, Goddard DR, Colmerauer ME, Davis CE (1985) Effect of theophylline on differentiation of *Trypanosoma brucei*. *Infect Immun* 49:844–847
- Seebeck T, Schaub R, Johnner A (2004) cAMP signalling in the kinetoplastid protozoa. *Curr Mol Med* 4:585–599
- Shalaby T, Liniger M, Seebeck T (2001) The regulatory subunit of a cGMP-regulated protein kinase A of *Trypanosoma brucei*. *Eur J Biochem* 268:6197–6206
- Siman-Tov MM, Aly R, Shapira M, Jaffe CL (1996) Cloning from *Leishmania major* of a developmentally regulated gene, c-lpk2, for the catalytic subunit of the cAMP-dependent protein kinase. *Mol Biochem Parasitol* 77:201–215
- Strickler JE, Patton CL (1975) Adenosine 3', 5'-monophosphate in reproducing and differentiated trypanosomes. *Science* 190:1110–1112
- Stuart K, Brun R, Croft S, Fairlamb A, Gurtler RE, McKerrow J, Reed S, Tarleton R (2008) Kinetoplastids: related protozoan pathogens, different diseases. *J Clin Invest* 118:1301–1310
- Tyler K, Miles M (2002) World class parasites: volume 7 American trypanosomiasis. Kluwer Academic Publishers, Boston
- Urbina JA (2010) Specific chemotherapy of Chagas disease: relevance, current limitations and new approaches. *Acta Trop* 115(1–2):55–68
- Vassella E, Reuner B, Yutzy B, Boshart M (1997) Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. *J Cell Sci* 110(Pt 21):2661–2671
- Walter RD (1974) 3':5'-cyclic-AMP phosphodiesterase from *Trypanosoma gambiense*. *Hoppe Seylers Z Physiol Chem* 355:1443–1450
- Walter RD, Buse E, Ebert F (1978) Effect of cyclic AMP on transformation and proliferation of leishmania cells. *Tropenmed Parasitol* 29:439–442
- Wang H, Liu Y, Huai Q, Cai J, Zoraghi R, Francis SH, Corbin JD, Robinson H, Xin Z, Lin G, Ke H (2006) Multiple conformations of phosphodiesterase-5: implications for enzyme function and drug development. *J Biol Chem* 281:21469–21479
- Wang H, Yan Z, Geng J, Kunz S, Seebeck T, Ke H (2007) Crystal structure of the *Leishmania major* phosphodiesterase LmjPDEB1 and insight into the design of the parasite-selective inhibitors. *Mol Microbiol* 66:1029–1038
- Wang H, Yan Z, Yang S, Cai J, Robinson H, Ke H (2008) Kinetic and structural studies of phosphodiesterase-8A and implication on the inhibitor selectivity. *Biochemistry* 47:12760–12768
- Weatherly DB, Boehlke C, Tarleton RL (2009) Chromosome level assembly of the hybrid *Trypanosoma cruzi* genome. *BMC Genomics* 10:255
- Zoraghi R, Seebeck T (2002) The cAMP-specific phosphodiesterase TbPDE2C is an essential enzyme in bloodstream form *Trypanosoma brucei*. *Proc Natl Acad Sci USA* 99:4343–4348
- Zoraghi R, Kunz S, Gong K, Seebeck T (2001) Characterization of TbPDE2A, a novel cyclic nucleotide-specific phosphodiesterase from the protozoan parasite *Trypanosoma brucei*. *J Biol Chem* 276:11559–11566

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