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Harald H. H. W. Schmidt
Franz Hofmann
Johannes-Peter Stasch
Editors

cGMP: Generators, Effectors and Therapeutic Implications

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cGMP: Generators, Effectors and Therapeutic Implications

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Preface

This book is an enthusiastic celebration of cyclic guanosine monophosphate (cGMP) and amply illustrates the importance of this field of science to patients and how it has evolved. Cyclic GMP, until recently the forgotten sibling of cyclic adenosine monophosphate (cAMP), was discovered as a second messenger in the late 1960s. For many years, cGMP retained the status of a sleeping princess. Indeed, the biological role of cGMP was unknown until the 1980s, when two key discoveries were made. First, it was found that natriuretic peptides (NP) could stimulate cGMP synthesis by binding to the particulate guanylate cyclase (pGC). Second, it became apparent that nitrovasodilators such as glycerol trinitrate, which liberate nitric oxide (NO), are potent activators of the cGMP-forming signaling enzyme, soluble guanylate cyclase (sGC). After the discovery of endogenous NO formation in the late 1980s and the 1998 Nobel Prize in Physiology or Medicine, many researchers and physicians again became interested in the NO/sGC interaction and cGMP-dependent signaling.

Cyclic GMP plays a key role in the regulation of cardiovascular homeostasis including smooth muscle relaxation, platelet inhibition and vascular growth and differentiation, and we are beginning to understand the respective molecular mechanisms downstream of cGMP. Subsequently, the effector systems of cGMP - protein kinases, ion channels and phosphodiesterases - have emerged as sub-fields of cGMP research and therapeutic targets. Since this complexity of cGMP formation, metabolism and effectors now underscore the importance of cGMP signaling, it is time to integrate all aspects of cGMP for the first time in one book, about half a century after its discovery.

This book is exclusively devoted to this exciting and important signaling molecule, addressing all recent advances in understanding guanylate cyclase regulation, NO/sGC interactions, cGMP effector mechanisms and their pathophysiological and pharmacological implications. Particular attention will also be given to clinical applications of the novel cGMP-elevating drugs which are on the horizon, thus spanning the continuum from basic science to clinic.

The first part of the book deals with the basic generators and effectors of cGMP. The middle section explores how pharmacologically active compounds can expand

our understanding of physiology and pathophysiology. The concluding section surveys the relevance of cGMP to disease, and therapeutic applications of novel cGMP-elevating compounds.

There is tremendous promise behind cGMP itself, as well as the numerous other molecules and processes associated with the NO/sGC/cGMP and NP/pGC/cGMP pathways. Collaborative efforts among biochemists, physiologists, pharmacologists and clinicians are the key in realizing this promise. Undoubtedly, exciting times lie ahead for the GC field.

If this book helps to boost further the rapidly evolving cGMP field, which is now undergoing a transition from basic science to clinical applications, to improve care and treatment of patients, the efforts of the editors and our editorial assistant Susanne Dathe will not have been in vain.

Melbourne *H.H.H.W. Schmidt*
München *F. Hofmann*
Wuppertal *J.-P. Stasch*

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A Short History of cGMP, Guanylyl Cyclases, and cGMP-Dependent Protein Kinases

Alexander Y. Kots, Emil Martin, Iraida G. Sharina, and Ferid Murad

"I have but one lamp by which my feet are guided, and that is the lamp of experience. I know no way of judging of the future but by the past." Edward Gibbon

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Abstract Here, we review the early studies on cGMP, guanylyl cyclases, and cGMP-dependent protein kinases to facilitate understanding of development of this exciting but complex field of research encompassing pharmacology, biochemistry, physiology, and molecular biology of these important regulatory molecules.

Keywords: Guanylyl cyclase · cGMP · Protein kinase G · Phosphodiesterase

1 Introduction

This chapter describes a number of historical aspects of discoveries made in the fields of cGMP research. To facilitate the understanding of signaling pathways, we have included a general scheme of these pathways in Fig. 1.

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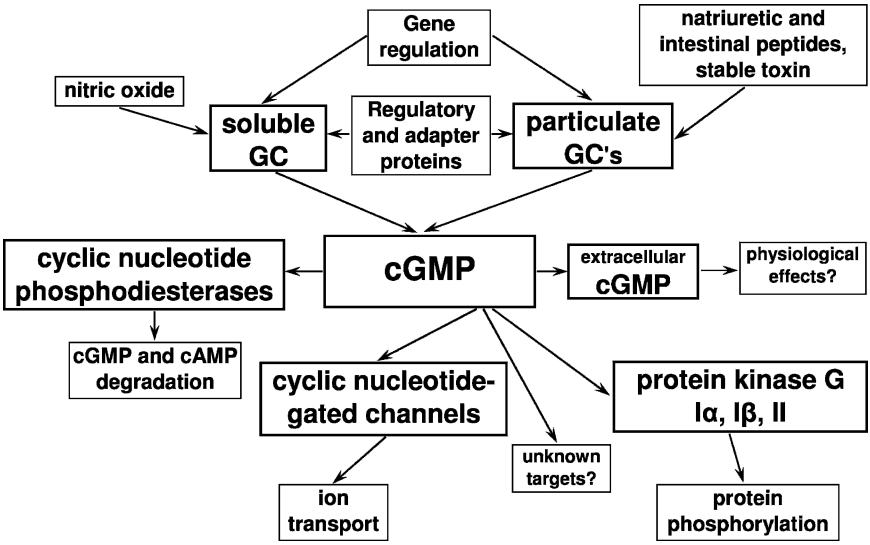

Fig. 1 Scheme of cGMP signaling pathways. Soluble and particulate guanylyl cyclases (GC) are stimulated mainly by nitric oxide and natriuretic and intestinal peptides and stable toxin from *Escherichia coli*, respectively. These enzyme are known to be regulated on the expression level (gene regulation) and by certain regulatory and adapter proteins. They synthesize cGMP, which can be degraded by cGMP-specific phosphodiesterases or transported to the extracellular environment via specific mechanism. The main targets of cGMP-dependent regulation include cyclic nucleotide phosphodiesterases (cAMP- and cGMP-specific), cyclic nucleotide-gated ion channels and cGMP-dependent protein kinases (protein kinase G)

The primary scope of this chapter is to outline the main events in the field of cGMP, but not to cite every important publication. The authors sincerely apologize to all scientists whose important papers are not cited here due to size limitations.

2 Discovery of cGMP, Guanylyl Cyclases, and cGMP Phosphodiesterases

Since the discovery of cAMP (Rall and Sutherland 1958; Sutherland and Rall 1958), it had become evident that other $3', 5'$ cyclic nucleotides might exist and that these compounds might be important for regulation of cellular physiology. In 1960, cGMP was synthesized for the first time (Smith et al. 1961) and synthetic compound was shown to be degraded by enzymatic hydrolysis similar to cAMP (Drummond and Perrott-Yee 1961). An enzyme capable of digesting the $3'$–$5'$ phosphodiester bond of cGMP was detected also by Kuriyama et al. (1964). At about same time, existence of endogenously produced cGMP was confirmed by isolation and identification of cGMP from rabbit urine (Ashman et al. 1963). Later, this was confirmed by another study from the same laboratory (Price et al. 1967), which analyzed specific activity of urinary cGMP in rats treated with [^{32}P]phosphate and suggested that cGMP is

synthesized in a reaction catalyzed by a cyclase, which might be similar to previously discovered adenylyl cyclase. In the laboratory of Earl Sutherland, hormonal regulation of urinary cGMP levels was investigated and it was found that a number of steroid, thyroid, and pituitary hormones can regulate production of cGMP in rats (Hardman et al. 1966). This work was paralleled by a study in which a phosphodiesterase specific for cAMP and cGMP was isolated and partially purified from dog heart (Nair 1966) with subsequent publications revealing more insight into cGMP-specific phosphodiesterases (Beavo et al. 1970; Cheung 1971; Kakiuchi et al. 1971; Thompson and Appleman 1971). At this point, considering early publications on cGMP secretion (Hardman et al. 1966; Broadus et al. 1971; also reviewed by Sager 2004), it became apparent that steady state levels of cGMP in cells and tissues are determined by the balance of cGMP synthesis by guanylyl cyclases and cGMP extrusion out of the cell and degradation by phosphodiesterases. It was also demonstrated that cGMP has certain pharmacological effects (for example, see Murad et al. 1970) and later studies pointed to possible regulation of cGMP levels by various hormones (again, just an example, Kimura et al. 1974).

Rapid progress in the field was possible because of the development of an accurate radioimmunoassay for cGMP (Brooker et al. 1968). In 1969, guanylyl cyclase activity was described (Bohme et al. 1969; Hardman and Sutherland 1969; Schultz et al. 1969; White and Aurbach 1969).

At the same time, it also became apparent that guanylyl cyclases exist as a number of isoforms, some of which are soluble while others are membrane-bound or might associate with the cytoskeleton (Goridis and Morgan 1973; Kimura and Murad 1974, 1975a, b). In this case, the situation was quite different from adenylyl cyclase research. Hormones and mediators capable of activating adenylyl cyclase have been known for quite a while, whereas what physiologically relevant molecules induce activation of guanylyl cyclases remained quite a mystery. Earl Sutherland wrote in his Nobel lecture in 1971 comparing the research areas of cAMP and cGMP: "Then (implying cAMP) we had a function, and found a nucleotide; now (implying cGMP) we have a nucleotide, and are trying to discover its function" (published online at nobelprize.org).

Several hormones and neuromediators were described to influence the activities of guanylyl cyclases in intact cells, but there was no direct evidence of activation of this enzyme. The problem at that time was that some key regulators were not yet discovered. And the routes to their discoveries took several years and were not always straightforward. Exciting examples of such mediators are nitric oxide, endothelium-derived relaxing factor (EDRF), and atriopeptins.

3 History of Soluble Guanylyl Cyclase

There is an abundant literature on the subject of nitric oxide as endogenous messenger and for more detailed description of this topic, the readers are referred to other publications (for example, see Murad et al. 1978; Moncada 1990; Murad 1998, 1999, 2006; Yetik-Anacak and Catravas 2006).

Essentially, the mystery of soluble guanylyl cyclase and nitric oxide can be split in several key issues. First, during early studies, it was shown that calcium infusions stimulate excretions of cGMP and not cAMP with urine (Kaminsky et al. 1970). In addition to this, it was shown that acetylcholine can increase the levels of cGMP indirectly in isolated perfused rat heart (George et al. 1970) and that calcium is important for regulation of cGMP levels in tissues (Schultz et al. 1973). Another aspect of the puzzle involved activation of the soluble isoform of guanylyl cyclase by nitric oxide, which can be generated from other activators such as azide, nitrate, and hydroxylamine (Kimura et al. 1975a, b) in the presence of catalase and by nitrovasodilators (Arnold et al. 1977; Katsuki et al. 1977). These two aspects seemed to be completely unrelated to each other and they remained so for another 10 years. Meanwhile, relaxation of blood vessels by acetylcholine was shown to be endothelium-dependent and to involve another mysterious factor, EDRF, which acted on an unidentified target in smooth muscle cells (Furchgott and Zawadzki 1980). It was soon shown that EDRF increases cGMP synthesis in isolated blood vessels and increases protein phosphorylation in smooth muscle (Rapoport et al. 1983; Rapoport and Murad 1983). Later on, it was suggested that EDRF can be an endogenous nitrovasodilator (Murad 1986). This was subsequently followed by physiological and biochemical studies showing a very close chemical and pharmacological similarity of EDRF and nitric oxide (reviewed in Ignarro 1989). On the other hand, previously, it was shown that L-arginine could activate crude or partially purified soluble guanylyl cyclase but had no effect on purified enzyme, (Deguchi and Yoshioka 1982) which indicated that partially purified enzyme preparation converted L-arginine to an activator, presumably being nitric oxide. These findings were quite rapidly followed by a number of publications identifying endogenous production of nitric oxide that is catalyzed by a family of NO-synthases, which use arginine as a substrate (early reviews on the subject are still quite comprehensive; Forstermann et al. 1991; Moncada and Higgs 1991; Bredt and Snyder 1992; Stuehr and Griffith 1992).

Thus, it took about 15 years to understand why calcium and acetylcholine increase the production of cGMP. At present, this signaling pathway is considered quite established and it is described in all textbooks on vascular pharmacology. A rather simplified and schematic understanding is that acetylcholine binds to muscarinic receptors on endothelial cells and this elevates the intracellular calcium concentration, which leads to activation of nitric oxide synthesis due to calmodulin dependency of NO-synthase in endothelial cells. NO then diffuses into smooth muscle cells and activates soluble guanylyl cyclase increasing the level of cGMP causing smooth muscle relaxation. The relaxation mechanism might involve decreased levels of cytosolic calcium in smooth muscle and decreased phosphorylation of myosin light chain kinase caused by cGMP (Murad 1986, 1998, 2006) and this in turn might be influenced via modulation of myosin light chain phosphatase in case of cGMP-induced calcium desensitization (Khromov et al. 2006).

In parallel to these exciting discoveries, the enzymology of soluble guanylyl cyclase was studied and the structure of the enzyme was determined due to combined efforts of various research teams. Again, the reader is referred to a number

of excellent detailed reviews on these subjects (for example see Lucas et al. 2000). Critical findings include enzyme purification from a number of laboratories and demonstration of its heterodimeric nature (Kamisaki et al. 1986) and the presence of a prosthetic heme moiety, which is required for stimulation by nitric oxide (Gerzer et al. 1981; Ignarro et al. 1982a).

Of special note is the activation of soluble guanylyl cyclase by protoporphyrin IX (Ignarro et al. 1982b) suggesting that the enzyme can be stimulated by replacement of the heme. This concept was further advanced by development of two highly potent synthetic activators of heme-deficient or oxidized enzyme, BAY58-2667 (Stasch et al. 2002) and HM1766 (Schindler et al. 2006), and by studies on vasorelaxant and cGMP-elevating effects of delta-aminolevulinic acid, a natural heme precursor (Mingone et al. 2006).

Cloning and deduced primary structures of the beta subunit of the enzyme were reported (Koesling et al. 1988; Nakane et al. 1988) soon followed by the primary structure of the alpha1 subunit (Koesling et al. 1990; Nakane et al. 1990) and alpha2 subunit (Harteneck et al. 1991) and more recent reports on genomic organization of alpha1 and beta1 subunits of the enzyme (Sharina et al. 2000) and promoter regulation for the alpha and beta subunits (Sharina et al. 2003). However, the exact mechanism of activation of the enzyme by nitric oxide and determination of the X-ray structure of the protein are still a matter of research efforts by many investigators.

4 History of Particulate Guanylyl Cyclases

Again, the readers are referred to comprehensive historical and mechanistic reviews of this subject (e.g. Schulz et al. 1989; Lucas et al. 2000; regarding retinal enzymes see Pugh et al. 1997). The situation in this case was similar to the soluble isoform. It was known that particulate enzymes are regulatory; however, their activators were not identified in the early 70s.

First report on specific stimulation of particulate guanylyl cyclase showed stimulation of enzyme activity in the intestinal tissue by stable toxin of *Escherichia coli* (Hughes et al. 1978). Only 14 years later, the endogenous peptides guanylin and uroguanylin were isolated and demonstrated to be the activator of particulate guanylyl cyclase type C (Currie et al. 1992; Hamra et al. 1993) soon after the primary structure of the enzyme was determined and its functional role as a stable toxin receptor was confirmed (Schulz et al. 1990). Later, this was re-confirmed in knockout mice (Mann et al. 1997).

In case of other particulate guanylyl cyclases, great progress was achieved mostly due to studies of sea urchin spermatozoa, where a number of peptides were identified as stimulators of enzyme activity (for example, see Hansbrough and Garbers 1981). At the same time, peptides of somewhat similar structure were identified as natriuretic factors in atrial myocardial extracts (atrial natriuretic peptides; de Bold et al. 1981). These peptides were shown to be endogenous activators of particulate guanylyl cyclase in various tissues (Hamet et al. 1984; Waldman et al. 1984;

Winquist et al. 1984). Later on, co-purification (Kuno et al. 1986; Paul et al. 1987), molecular cloning, sequencing, and expression of recombinant protein indicated that natriuretic peptide receptor is guanylyl cyclase type A (Singh et al. 1988; Chinkers et al. 1989; Lowe et al. 1989; Thorpe and Garbers 1989). The presence (Leitman et al. 1986a, b) and structure of the clearance receptor, which lacks the guanylyl cyclase domain and includes only natriuretic factor-binding region, was also determined (Fuller et al. 1988). The emerging new family of hormonal messengers was complemented by discovery of two other molecules, C-type natriuretic peptide (Furuya et al. 1990) stimulating guanylyl cyclase type B (Koller et al. 1991) and brain natriuretic peptide (Song et al. 1988; Sudoh et al. 1988).

In retina, cGMP-specific phosphodiesterase (Pannbacker et al. 1972) and guanylyl cyclase (Pannbacker 1973) were described for the first time in the early 70s but the role of cGMP in regulation of photoreceptor signal transduction was controversial for a long time. The enzyme in retina does not appear to be regulated by extracellular mediators unlike other known guanylyl cyclases but is modulated by intracellular messengers and proteins. Some researchers argued that calcium ions play the critical role in phototransduction whereas others insisted that cGMP is more important (reviewed in Pugh et al. 1997). Eventually, it was demonstrated that ion channels of the frog outer segments are regulated by cGMP and not by calcium (Fesenko et al. 1985) and the argument was essentially concluded after a report on submicromolar calcium regulation of rod guanylyl cyclase activity (Pepe et al. 1986). The study was further expanded in 1988 (Koch and Stryer 1988) and it became apparent that calcium-dependent regulation of guanylyl cyclase and light-dependent regulation of cGMP-specific phosphodiesterase are tightly coupled during the course of phototransduction.

The search for the activators of particulate guanylyl cyclases is not yet over since there are two so-called orphan transmembrane enzymes with unknown activators, guanylyl cyclases types D and G. Recent encouraging progress had been made with identification of guanylin and/or uroguanylin as regulators of guanylyl cyclase type D, thus contributing to chemosensory function in olfactory epithelium (Leinders-Zufall et al. 2007) while regulation of the enzyme by intracellular calcium and calcium-binding proteins is also important for odorant signal transduction (Duda and Sharma 2008).

Interestingly, cloning studies with the alpha and beta subunits of soluble guanylyl cyclase, the particulate isoforms, and adenylyl cyclase have revealed considerable homology in the catalytic domains of all of these enzymes. Upon activation, soluble guanylyl cyclase can synthesize cGMP and cAMP (Mittal and Murad 1977; Mittal et al. 1979). Mutation of a few amino acids in the catalytic domain can also change the nucleotide substrate specificity to make either cyclic nucleotide (Sunahara et al. 1998). Presumably, the catalytic region of adenylyl and guanylyl cyclases originated from a common ancestral gene that fused with various regulatory domains and thus diverged in evolution (Beuve 1999; Kasahara et al. 2001).

5 History of cGMP-Dependent Protein Kinases (PKG)

Appreciation of cGMP as an intracellular second messenger was followed by search for a cGMP-dependent kinase similar to what had been discovered in case of cAMP. Surveying various tissues and species, Kuo and Greengand found that partially purified protein kinase from lobster tail was activated by cAMP and also by cGMP in a histone phosphorylation assay (Kuo and Greengard 1969). In other tissues, stimulation by cAMP was more effective. In a follow-up study they were able to chromatographically separate these two activities and provided a clear evidence for a separate cGMP-regulated protein kinase (Kuo and Greengard 1970), which is weakly activated by cAMP.

5.1 Purification and Structure of cGMP-Dependent Protein Kinase

Following this discovery, the presence of cGMP-dependent protein kinase activity was demonstrated in various tissues (Hofmann and Sold 1972; Sold and Hofmann 1974) and a number of scientists tried to purify PKG (for example Kuo and Greengard 1974; Nakazawa and Sano 1975). In 1976, Gill and coworkers used an immobilized cGMP analog to affinity capture the cGMP-dependent kinase, which was eluted by cGMP to obtain a homogeneous preparation (Gill et al. 1976). Purified enzyme was a homodimer (Lincoln et al. 1977). In subsequent studies, the affinity purification procedure was modified to use a more accessible cAMP-affinity capture (Glass and Krebs 1979) which provided for a lower level of contamination with cAMP-dependent kinases. Interestingly, the early assays for cGMP were based on radioimmunoassay and the method was similar to previous development of a radioimmunoassay for cAMP. Following the report on cGMP-dependent protein kinase (Kuo and Greengard 1970), lobster muscle extract was utilized for a cGMP binding assay (Murad and Gilman 1971; Murad et al. 1971).

Once the purification procedure was established, several groups focused on determining the primary structure of the enzyme. Amino acid composition of PKG and PKA preparations is similar suggesting a high degree of homology of these two enzymes (Lincoln and Corbin 1977). First peptide sequence of the ATP-binding site of bovine cGMP-dependent kinase was reported in 1982 (Hashimoto et al. 1982). Shortly after that, primary sequence of a peptide from the “hinge” domain and its autophosphorylation site were sequenced (Takio et al. 1983). In 1984, Titani and coworkers finally obtained sufficient quantities of the enzyme to generate overlapping peptide fragments and identified the first full length sequence of PKG (Takio et al. 1984) confirming high degree of similarity with cAMP-dependent protein kinase.

5.2 Discovering the Diversity of PKG Isoforms

Investigating the PKG enzyme from bovine aorta smooth muscle, Wolfe and coworkers noticed two chromatographically close but distinct peaks (Wolfe et al. 1987, 1989). One of these peaks was identical to previously described enzyme purified from bovine lung and was designated as type Iα. The second peak contained a protein with a different N-terminal segment and was designated as type Iβ. Since the difference was only at the N-terminus, it was suggested that these types might result from splicing and are not the products of different genes. In 1989, Wernet from the Hofmann group screened a cDNA library form bovine trachea smooth muscle and isolated two independent cDNAs encoding for the type Iα and Iβ isoforms of cGMP-dependent kinase (Wernet et al. 1989). These cDNAs apparently arise from the same gene through alternative splicing.

Several years earlier, while investigating the distribution of cGMP-dependent protein kinase activity in the intestinal tissue, de Jonge found a membrane-associated activity in the brush border of rat and pig intestine (de Jonge 1981). This activity was biochemically distinct from already well-characterized PKG activity of the enzyme purified from the lung. It associated with the membrane and had a different molecular size (86 vs 74 kDa monomer). This suggested that a novel isoform of cGMP-dependent kinase exists in intestinal tissue. Investigating PKG genes in Drosophila, Kalderon and Rubin found a proof that two independent genes encode for different homologous PKG proteins (Kalderon and Rubin 1989). Inspired by these findings, Uhler screened the cDNA library form mouse brain and found two different types of cDNA. One encoded for the type I enzyme, while another encoded for a novel cGMP-dependent kinase (Uhler 1993) designated as type II. Walter and colleagues screened a cDNA library from rat intestine and found a cDNA for type II enzyme confirming that the membrane-associated PKG discovered in the intestinal brush border is indeed an independent isoform (Jarchau et al. 1994). Although, originally described as a monomer of 86 kDa (de Jonge 1981), type II cGMP-dependent kinase was later shown to be a dimer (Gamm et al. 1995) similar to type I enzyme.

6 Conclusion

The characterization of the proteins functioning in cyclic nucleotide synthesis, hydrolysis, and downstream physiological effects, particularly cGMP, revealed these proteins as important macromolecular targets for drug discovery and development. Many novel drugs have been and will be developed for therapies of a host of important disorders which might explain the growing popularity of this field. The pharmacological applications of these discoveries drive this whole field of research forward at a considerable speed already for over 40 years and will continue to inspire the scientists to contribute more exciting discoveries to improve our understanding of biological processes associated with cGMP.

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Part I
Basic Generators and Effectors

Biochemistry of Soluble Guanylate Cyclase

Emily R. Derbyshire and Michael A. Marletta

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Abstract Nitric oxide (NO) functions in biology as both a critical cytotoxic agent and an essential signaling molecule. The toxicity of the diatomic gas has long been accepted; however, it was not known to be a signaling molecule until it was identified as the endothelium-derived relaxing factor (EDRF). Since this discovery, the physiological signaling pathways that are regulated by NO have been the focus of numerous studies. Many of the cellular responses that NO modulates are mediated by the heme protein soluble guanylate cyclase (sGC). NO binds to sGC at a diffusion controlled rate, and leads to a several 100-fold increase in the synthesis of the second messenger cGMP from GTP. Other diatomic gases either do not bind (dioxygen), or do not significantly activate (carbon monoxide) sGC. This provides selectivity and efficiency for NO even in an aerobic environment, which is critical due to the high reactivity of NO. Several biochemical studies have focused on elucidating the mechanism of NO activation and O_2 discrimination. Significant advances in our understanding of these topics have occurred with the identification and characterization of the sGC-like homologues termed Heme-Nitric oxide and OXygen binding (H-NOX) proteins.

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1 The NO/cGMP Signaling Pathway

In eukaryotes nitric oxide synthase (NOS) produces NO from L-arginine (reviewed in (Alderton et al. 2001; Dudzinski et al. 2006; Stuehr 1997)). There are three isoforms of NOS, endothelial, neuronal and inducible (eNOS, nNOS, and iNOS). Both eNOS and nNOS are constitutively expressed and iNOS is induced with the appropriate immunostimulatory signals. The constitutive isoforms involved in signal transduction pathways (eNOS and nNOS) generate low nanomolar levels of NO and are regulated *in vivo* by the binding of calcium and calmodulin. NO produced by NOS can rapidly diffuse across a cell membrane to activate soluble guanylate cyclase (sGC), a hemoprotein that has evolved to selectively bind NO even in the presence of oxygen (μM) (Fig. 1). The sGC heme environment is unique when compared to the globins because it efficiently binds NO while having no affinity for O_2 coupled with an extremely slow rate of oxidation. sGC is a heterodimeric protein that consists of an α and a β subunit. NO binds to the heme of sGC and leads to a several 100-fold increase in the synthesis of cGMP from GTP. In addition to NO, cGMP production by sGC is regulated allosterically by substrate GTP and by ATP (Cary et al. 2005; Ruiz-Stewart et al. 2004) which are present at ~0.2 and ~1.7 mM, respectively, *in vivo* (Traut 1994). The efficient binding of NO by sGC allows for the rapid production of cGMP which then binds to phosphodiesterases (PDE), ion-gated channels, and cGMP-dependent protein kinases (cGK) to regulate several physiological functions including vasodilation, platelet aggregation and neurotransmission (Munzel et al. 2003; Sanders et al. 1992; Warner et al. 1994).

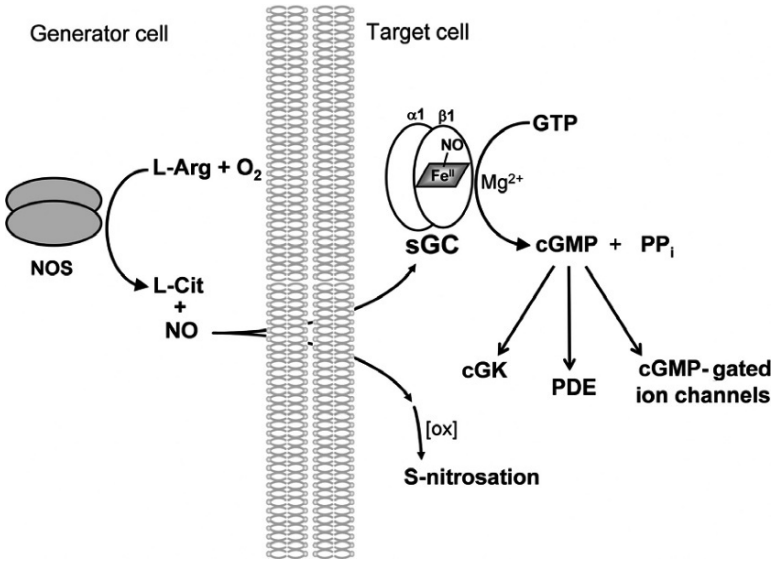

Fig. 1 Nitric oxide signal transduction pathway. NO synthesized by NOS diffuses across cell membranes to a target cell. NO activates sGC, which leads to an increase in cGMP synthesis. The oxidation products of NO can also react with protein thiols, which leads to protein *S*-nitrosation.

Both NOS and sGC are critical to the regulation of homeostasis, and dysfunction in the NO signaling pathway has been linked to several diseases (reviewed in (Bredt 1999)). The importance of the NO signaling pathway has also been demonstrated in mouse models where the triple NOS knock-outs exhibit characteristics consistent with nephrogenic diabetes insipidus (Morishita et al. 2005). Knock-outs of the sGC β1 subunit exhibit elevated blood pressure, reduced heart rate and dysfunction in gastrointestinal contractility (Friebe et al. 2007), and studies on mice deficient of the sGC α1 subunit indicate that the protein is essential for NO-mediated pulmonary vasodilation (Vermeersch et al. 2007).

2 sGC Isoforms

sGC is a heterodimeric protein consisting of two homologous subunits, α and β. The most commonly studied isoform is the α1β1 protein; however, α2 and β2 subunits have also been identified (Harteneck et al. 1991; Yuen et al. 1990). The α subunits are highly homologous with 48% sequence identity, and β subunits have an overall sequence identity of 41%. Generally, a higher degree of variability is observed between α subunits of different species when compared to β subunits.

The localization of each subunit has been studied in mammals including humans, rats, and cows. Both α1 and β1 are expressed in most tissues and it is well accepted that these proteins form a physiologically relevant heterodimer (Budworth et al. 1999). By Western blotting and quantitative PCR analysis the α2 subunit is found in fewer tissues compared to the α1 and β1 isoforms, but is highly expressed in the brain, lung, colon, heart, spleen, uterus and placenta (Bellingham and Evans 2007; Budworth et al. 1999; Russwurm et al. 1998). Studies with the purified protein have shown that the α2β1 heterodimer exhibits ligand binding characteristics identical to the α1β1 heterodimer (Russwurm et al. 1998), but a splice variant of the α2 subunit (α2i) dimerizes with the β1 subunit to form an inactive complex. α2i contains an in-frame insertion of 31 amino acids within the catalytic domain and appears to function as a dominant negative protein (Behrends et al. 1995). The β2 isoform is not very prevalent and is expressed primarily in the kidney (Yuen et al. 1990). While the β2 isoform has not yet been purified and characterized, transient expression of the full-length protein in insect cells show that it is active as a homodimer, but exhibits no cyclase activity when expressed with α1 (Koglin et al. 2001). However, further experiments are necessary to determine if β2 can indeed function as a novel homodimeric cyclase *in vivo*.

3 Architecture of sGC

The rat sGC α1 and β1 subunits are 690 and 619 amino acids in length, respectively. These proteins are part of a large family of sGC subunits that are conserved in eukaryotes. Generally there is the highest sequence variability at the N-terminus

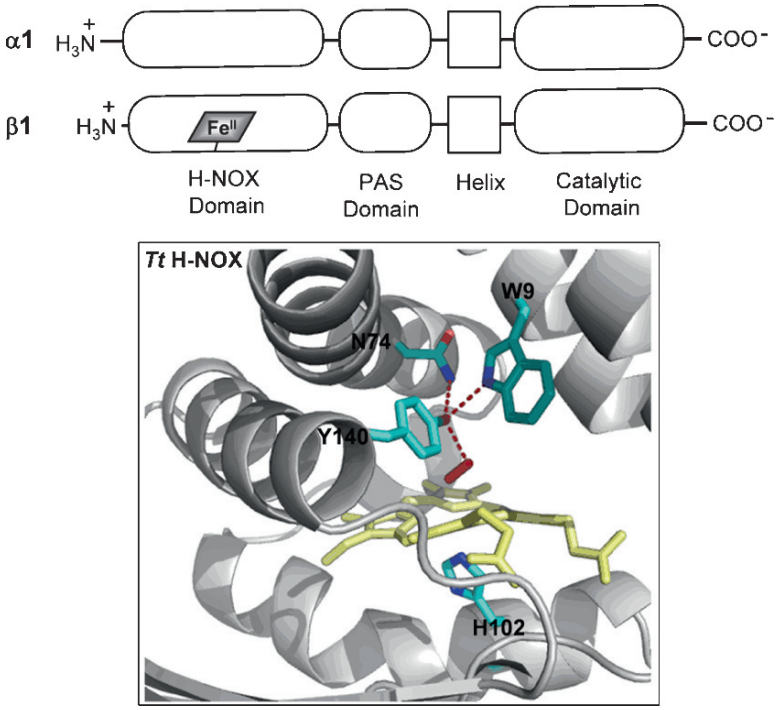

Fig. 2 Soluble guanylate cyclase. *Top panel*, domain architecture of sGC. sGC consists of two homologous subunits, α1 and β1. Each subunit contains an N-terminal H-NOX domain, a central PAS domain, a putative amphipathic helix, and a C-terminal catalytic domain. Heme (grey parallelogram) binds to the H-NOX domain on the β1 subunit. *Bottom panel*, crystal structure of *Tt* H-NOX bound to O_2 (1U55.pdb). Residues that stabilize O_2–binding (W9, N74 and Y140) and coordinate to the heme iron (H102) are shown.

of α subunits and the greatest sequence identity at the C-terminus of both the α and β proteins. Each sGC subunit consists of four distinct domains. The β1 subunit contains a N-terminal heme binding domain, a Per/Arnt/Sim (PAS) domain, a putative amphipathic helix, and a C-terminal catalytic domain (reviewed in (Cary et al. 2006)) (Fig. 2). While bioinformatics have sufficiently identified the PAS domain, the helix, and catalytic domain, experiments with sGC truncations and site-direct mutagenesis were necessary to localize the minimal heme binding domain of sGC. These experiments involved the systematic mutation of histidines (Wedel et al. 1994), expression of various truncations in *E. coli* (Zhao and Marletta 1997) and deletion of the β1 N-terminus (Wedel et al. 1995). Taken together, these studies showed that the β1 N-terminus constituted the heme binding domain and suggested that histidine 105 (rat numbering) was the proximal heme ligand.

The sGC heme binding domain was initially localized to residues 1–385 (Zhao and Marletta 1997); however, it is now clear that residues 1 to ∼194 encode the minimal heme binding domain (Karow et al. 2005). While it was once thought that

this domain was exclusively associated with NO activated guanylate cyclases found in mammals, it is now known that the sGC heme domain is part of a conserved family of proteins found in prokaryotes and eukaryotes (Iyer et al. 2003). This family of proteins is termed the Heme-Nitric oxide and OXygen binding family (H-NOX) based on their ligand binding properties and are discussed in more detail in the next section (reviewed in (Boon and Marletta 2005)).

The central region of sGC contains two domains of unknown function. One domain includes residues ~200–350 of β1 and ~270–400 of α1, and is predicted to adopt a PAS-like fold (Kelley et al. 2000). Typically PAS domains mediate protein-protein interactions and/or bind small molecules such as hemes, flavins and nucleotides. The other domain, a putative helix region, appears to be unique to sGC and shares no homology with any other protein in the NCBI protein database. Site-directed mutagenesis of residues in this region on the α1 subunit (Shiga and Suzuki 2005), as well as a bimolecular fluorescence complementation assay in cells suggest that the central regions of both sGC subunits are important for the formation of a functional heterodimer (Rothkegel et al. 2007).

The catalytic domains have been localized to the C-terminal 467–690 and 414–619 residues of the α1 and β1 subunits, respectively (Winger and Marletta 2005). These domains must form a heterodimer for cGMP to be synthesized, and in the full-length protein the catalytic efficiency of the protein is dependent on the heme ligation state of the β1 H-NOX domain. Interestingly, the activity of the isolated $\alpha1_{cat}\beta1_{cat}$ heterodimer is inhibited by the presence of the H-NOX domain (Winger and Marletta 2005). This shows that these domains interact in *trans*, and suggests that the NO mechanism of activation involves the relief of an inhibitory interaction between the H-NOX domain and the catalytic domains.

The sGC catalytic domains are highly homologous to both the particulate guanylate cyclase (pGC) and adenylate cyclase (AC) catalytic domains. There are crystal structures of AC catalytic domains (Sunahara et al. 1997; Zhang et al. 1997), but the structure of a guanylate cyclase has yet to be reported. However, key catalytic residues have been identified through multiple sequence alignments and homology models of the rat α1 and β1 catalytic domains based on the AC structures (Winger 2004). From these models two conserved aspartate residues on the α1 subunit (D485 and D529 rat numbering) are predicted to bind two Mg^{2+} ions. These residues are critical to catalysis as the associated metals likely function to activate both the nucleotide 3′-hydroxyl and the α phosphate for the reaction, and stabilize the charge on the β and γ phosphates on both substrate and product. Additionally, βN548 is proposed to orient the ribose ring for the reaction. Residues thought to be responsible for base recognition include E473 and C541 on the β1 subunit. Other residues on both α (R573) and β (R552) are thought to interact with the nucleotide triphosphate (Winger 2004). With the identification of these critical residues, predictions can be made about guanylate cyclase activity based on sequence analysis. This type of analysis would correctly predict that the β2 isoform can function as a homodimer but that β1, α1, and α2 need a partner to be active.

4 H-NOX Proteins

As mentioned earlier the sGC heme binding domain is now known to be part of a large family of heme binding proteins termed H-NOX proteins. To date all of the characterized bacterial H-NOX proteins bind heme, and were named H-NOX proteins to describe the ability of some of these proteins to bind O_2 in addition to NO and CO, while others, like sGC, discriminate against O_2 binding. H-NOX domains are found in both facultative aerobes and in obligate anaerobes. Interestingly, all of the isolated H-NOX domains from facultative aerobes do not bind O_2 while the H-NOX domains from obligate anaerobes form a stable O_2 complex. The variable ligand binding properties of these H-NOXs may have consequences for their ability to respond to different gases, and functional experiments will need to consider the possibility that the proteins may sense O_2 in addition to NO or CO.

In facultative aerobes the bacterial members of this family encode a single domain as a stand-alone protein, and genes that encode for either putative histidine kinases or diguanylate cyclases are found in the same predicted operon. This suggests that the domain has a role in two-component signaling in bacteria. In support of this hypothesis, an H-NOX domain and a predicted histidine kinase from *Shewanella oneidensis* were isolated and found to interact *in vitro*. Additionally, the functional interaction between the H-NOX and kinase was mediated by NO (Price et al. 2007).

In obligate anaerobes such as *Thermoanaerobacter tengcongensis* (*Tt*), the H-NOX domain is predicted to be part of a methyl-accepting chemotaxis protein. This would suggest that the H-NOX protein is involved in a chemotactic/signaling function, although this hypothesis awaits experimental verification. The isolation and characterization of the *Tt* H-NOX domain has significantly influenced our understanding of sGC since it was the first H-NOX domain to be structurally determined, and, moreover, it was crystallized bound to the diatomic ligand O_2 (Nioche et al. 2004; Pellicena et al. 2004) (Fig. 2). These structures showed that a distal pocket tyrosine (Y140) interacts with bound O_2 through a hydrogen bond, and provided the basis for the current molecular view of how sGC discriminates against O_2 binding, namely it lacks a hydrogen bond donor. This idea was further supported by the subsequent crystal structure of the O_2-excluding H-NOX from *Nostoc sp.* which shows that there is no hydrogen bond donor in the distal heme pocket (Ma et al. 2007). Additionally, comparison of the *Tt* H-NOX and *Ns* H-NOX structures in different ligation states (Fe^{II}-CO, Fe^{II}-NO and Fe^{II}-unligated states) provided evidence for a molecular mechanism of sGC activation. Specifically the differential pivoting and bending in the H-NOX heme upon NO or CO binding may account for the varying degree of activation induced by the two ligands (200-fold vs 4-fold, respectively) (Ma et al. 2007). The crystal structure of a domain from the *Nostoc punctiforme* signal transduction histidine kinase (STHK) was also recently determined. This domain has high sequence identity (35–38%) to the sGC PAS domain and the crystal structure showed that the domain dimerized and adopted a PAS fold (Ma et al. 2008). While structural studies of H-NOX proteins have facilitated our

understanding of sGC activation, the precise details about how movement in the sGC H-NOX domain affects the catalytic domain may remain unresolved until the full-length structure is elucidated.

Biochemical analyses on the sGC H-NOX domain, as well as the bacterial H-NOX proteins have also provided insight into the sGC heme environment and ligand binding characteristics – specifically discrimination against O_2 binding. If the *Tt* H-NOX distal pocket tyrosine is mutated to a leucine (Y140L), O_2 affinity is significantly reduced. Additionally, the introduction of a distal pocket tyrosine in O_2-excluding H-NOXs such as the *Legionella pneumophilia* H-NOX (*L*2 H-NOX) and the sGC β1(1–385) domain enables the proteins to bind O_2 (Boon et al. 2005).

5 sGC Activators

In addition to NO, a physiological sGC activator, CO can bind to the sGC heme and weakly activate the protein (Stone and Marletta 1994). The binding of CO leads to the formation of a 6-coordinate Fe^{II}-CO complex and a 2–4-fold increase in the rate of cGMP production. This activation is significantly lower than the 100–400-fold increase in cGMP production observed with NO. While the physiological relevance of activation of sGC by CO continues to be a matter of debate, CO has been used as a tool to probe the heme environment and activation properties of sGC. Other compounds that have been reported to activate sGC include organic nitrates (Artz et al. 2002) and protophoryrin IX (Ignarro et al. 1982). Conversely, reported inhibitors of sGC include heme and hematin (Ignarro et al. 1982), LY83583 (Mulsch et al. 1988), methylene blue (Dierks and Burstyn 1998), and the heme oxidant 1H-[1, 2, 4]oxadiazolol[4, 3-*a*]quinoxalin-1-one (ODQ) (Garthwaite et al. 1995).

It has become clear that small molecules can modulate the activity of sGC and that new therapeutics might be developed for the treatment of various diseases. Several compounds were screened for the ability to increase cGMP levels in cell lysates to search for a novel sGC activator. Such a screen led to the identification of YC-1, a benzylindazole derivative that activates sGC without coordinating to the heme (Ko et al. 1994). YC-1 only activates the Fe^{II}-unligated sGC state 2–4-fold, but significantly increases sGC activity when a ligand is bound at the Fe^{II} heme (Friebe et al. 1996; Stone and Marletta 1998). This synergistic activation leads to a Fe^{II}-CO complex that is activated 100–400-fold and a Fe^{II}-NO complex that is activated 200–800-fold. The novel sGC activator YC-1 led several groups to carry out structure-activity relationships to improve both the solubility and efficacy of YC-1. This led to the identification of several other compounds including BAY 41–2272, BAY 41–8543, CMF-1571, and A-350619 (reviewed in (Evgenov et al. 2006)). Collectively these molecules constitute a novel class of sGC activators that require the presence of the heme moiety and have the ability to synergistically activate sGC with both NO and CO.

5.1 Activator Binding

The binding of both NO and CO to the sGC heme has been extensively studied with standard spectroscopic methods such as electronic absorption, resonance Raman (RR) and electron paramagnetic resonance (EPR) to understand how they activate sGC. sGC is isolated in the reduced unligated state which is characterized by an absorbance maximum at 431 nm. While sGC has the same histidine ligated heme cofactor as found in the globins, it has distinct ligand binding properties. Unlike the globin heme, the sGC heme is stable in the presence of molecular oxygen and is not susceptible to oxidation. Both CO and NO bind to the reduced sGC heme and form complexes that are characterized by an absorbance maximum at 423 nm and 399 nm, respectively. Importantly, with these spectroscopic techniques ligand binding can be probed independently of activity, which is important for assessing the mechanism of sGC activation.

CO binds to the sGC heme with a fast on-rate $(3.58 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{s}^{-1})$ and a fast off-rate $(3.5\ \mathrm{s}^{-1})$ at 10°C. Experiments have shown that the binding of CO to the heme ($K_\mathrm{d} = 97\ \mu\mathrm{M}$) is directly correlated with sGC activation by CO ($K_\mathrm{A} = 24\ \mu\mathrm{M}$) (Stone and Marletta 1995). The binding and dissociation of CO from the sGC heme is increased in the presence of GTP and the allosteric activator YC-1 (Kharitonov et al. 1999). This effect shows that the binding of small molecules to a yet unknown site influences the heme site. This observation is also supported by experiments with RR spectroscopy which show that GTP and YC-1 have an effect on the RR spectrum of the sGC Fe^{II}-CO complex. Specifically, they cause a shift in both the Fe-CO and C-O stretching vibrations (Li et al. 2005; Makino et al. 2003; Martin et al. 2005). These effects are complicated as the small molecules change both the population of 6-coordinate Fe^{II}-CO and produce a small population of 5-coordinate Fe^{II}-CO. Some investigators have placed significant weight on the formation of a 5-coordinate Fe^{II}-CO complex and propose that the species is highly active. However, the Fe^{II}-CO complex in the presence of YC-1 and GTP is a mixture of three different species, and there is currently no evidence tying a specific activity with any one of these species.

Studies with NO have proven to be even more complicated. NO binds to the heme of sGC at a diffusion-controlled rate to form an initial 6-coordinate complex, which rapidly converts to a 5-coordinate ferrous nitrosyl complex (Stone and Marletta 1996). Initially it was thought to be a simple binding process, with a bimolecular formation of the 6-coordinate Fe^{II}-NO complex and a single exponential decay to the 5-coordinate Fe^{II}-NO complex. However, stopped-flow kinetics revealed that the NO association kinetics are best fit to a model that was bimolecular in NO concentration for both the formation of the 6-coordinate Fe^{II}-NO complex and, surprisingly, the conversion to the 5-coordinate Fe^{II}-NO complex (Stone and Marletta 1996; Zhao et al. 1999). The NO dependence of this second step suggests the existence of a second NO binding site (Stone and Marletta 1996; Zhao et al. 1999). Dissociation of NO from the heme is relatively slow $(0.0007\ \mathrm{s}^{-1})$ (Kharitonov et al. 1997) which would suggest that the sGC heme-NO complex has a 10–100 pM K_d. This is significantly lower than the measured K_d of NO activation

100–250 nM (Stone and Marletta 1996). This apparent contradiction was unexplained until recent work that showed NO coordination to the heme is not sufficient for full enzymatic activation (Cary et al. 2005; Russwurm and Koesling 2004). A low-activity Fe^{II}-NO complex can be formed in the presence of stoichiometric amounts of NO, and based on electronic absorption spectroscopy this species is identical to the highly active enzyme that is formed in the presence of substrate, products, YC-1, or excess NO. Based on these observations two mechanisms of NO activation have been proposed. One proposal is that excess NO activates the ferrous nitrosyl complex by binding to a nonheme site on the protein (Cary et al. 2005). The second proposal involves NO binding to the Fe^{II}-NO complex to form a transient dinitrosyl complex, which then converts to a 5-coordinate complex with NO bound in the proximal heme pocket (Russwurm and Koesling 2004) (Fig. 3). While both proposals involve the NO dependent conversion of a low-activity 5-coordinate Fe^{II}-NO complex to a high-activity 5-coordinate Fe^{II}-NO complex, the second proposal is dependent on NO binding to the heme cofactor. A recent study tested these proposals by using the heme ligand butyl isocyanide to block NO binding to the sGC heme. This study showed that NO activates the sGC Fe^{II}-butyl isocyanide complex without coordinating to the heme cofactor, and further supports the existence of a nonheme NO binding site (Derbyshire and Marletta 2007).

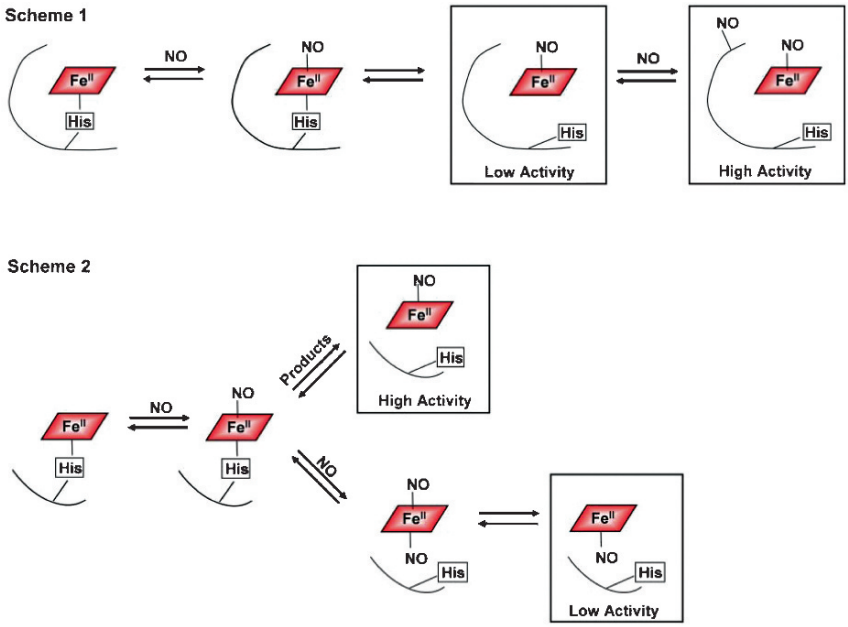

Fig. 3 Proposed mechanisms of NO activation of sGC. In Scheme 1, NO binds to the sGC heme to form a 5-coordinate ferrous nitrosyl complex. In the absence of excess NO this sGC species exhibits a low activity, which is activated by the binding of NO to a nonheme site. In Scheme 2, NO binding to the proximal heme pocket leads to a low-activity species, while NO binding to the distal heme pocket produces a high-activity species.

Recently the nitrosation of sGC in the presence of low levels of NO was reported. *S*-Nitrosation, the oxidative modification of cysteine residues, led to a reduction in NO-stimulated sGC activity (Sayed et al. 2007). This suggests that the modification may account for NO tolerance and desensitization. Additionally, this report highlights both the importance of cysteines to NO-induced activation, and shows that certain residues are accessible for binding NO.

As was observed for CO, the NO association and dissociation rates can be increased by the presence of GTP and YC-1 (Kharitonov et al. 1997; Russwurm and Koesling 2004; Winger et al. 2006). However, GTP only accelerates these rates if it is present before NO addition (Russwurm and Koesling 2004). This suggests there are multiple conformational changes that are dependent on the order of NO and GTP addition to sGC. Additionally, both RR and EPR spectroscopy confirm that the Fe^{II}-NO environment is influenced by the presence of GTP and YC-1. RR spectroscopy shows that the N-O stretch shifts from 1681 to 1700 cm^{-1} in the presence of GTP (Tomita et al. 1997), and the EPR spectrum of the Fe^{II}-NO complex shifts to a unique signal with either GTP or YC-1 addition (Derbyshire et al. 2008; Makino et al. 2003). This indicates that there are at least two sGC Fe^{II}-NO conformations of differing activities, and that the abundance of each conformation is influenced by both substrate and activator binding. This proposal is further supported by NO dissociation experiments which show that the sGC ferrous nitrosyl complex adopts two 5-coordinate conformations that are influenced by the presence of GTP and YC-1; a lower-activity complex which releases NO slowly and a higher-activity complex which releases NO rapidly (Winger et al. 2006). Understanding these molecular details of activation are critical for the rational design of therapeutic agents to treat diseases involving the NO/sGC/cGMP pathway.

6 Ligand Discrimination in sGC

The ability of sGC to select against O_2 binding is important for it to function as a NO sensor in aerobic environments such as human tissue. Some of the earliest proposals about ligand discrimination in sGC were based on RR data and a wealth of experiments carried out with the globins. These proposals include negative polarity in the heme distal pocket (Deinum et al. 1996), a weak Fe-His bond (Deinum et al. 1996) and a sterically crowded distal pocket (Jain and Chan 2003). All of these factors could reduce O_2 affinity in a protein; however, it is unlikely that one of these proposals could alone prevent O_2 from binding to the sGC heme. From structure activity relationships with sGC and structural investigations on the H-NOX proteins, it appears that sGC has a relatively large, hydrophobic distal heme pocket (Derbyshire et al. 2005; Nioche et al. 2004; Pellicena et al. 2004). Additionally, a comparison of the Fe-His bond strength in various O_2-excluding and O_2-binding H-NOXs shows that there is no obvious correlation between Fe-His bond strength and the ability of a protein to bind O_2. Clearly, studies on H-NOX proteins have discredited many of the leading hypotheses on ligand discrimination in sGC, but structural studies on H-NOXs provided an alternative explanation.

The crystal structure of the O_2-binding *Tt* H-NOX shows that a distal pocket tyrosine (Y140) interacts with bound O_2 though a hydrogen bond (Nioche et al. 2004; Pellicena et al. 2004). Multiple sequence alignments of other H-NOXs with this protein suggest that O_2-excluding H-NOXs, like sGC, have hydrophobic distal heme pockets that lack a hydrogen bond donor. This prediction is supported by the recently solved crystal structure of the O_2-excluding H-NOX from *Nostoc sp.* (Ma et al. 2007). Additionally, *L2* H-NOX and the sGC heme domain β1(1–385) were converted into O_2-binding proteins by introducing a single Tyr into their distal heme pockets (Boon et al. 2005). However, introduction of a single tyrosine in the full-length sGC did not stabilize O_2 binding, highlighting the importance of the other sGC domains in ligand binding (Martin et al. 2006). This work emphasizes the importance of a distal pocket hydrogen bond donor for ligand discrimination in H-NOXs, but suggests other factors must be critical for preventing O_2 binding in the mammalian sGCs. Recently, the identification of O_2-binding guanylate cyclases (*vide infra*) has allowed the importance of a distal pocket hydrogen bond donor in ligand discrimination to be more closely evaluated.

6.1 The Existence of O_2-Binding sGCs

As more genomes are sequenced an increasing number of organisms are found that contain predicted sGCs. Some sGCs are very similar to the well characterized rat α1 and β1 subunits while others vary significantly. For example, the *Drosophila melanogaster* genome contains five genes that code for sGCs. Two of these genes code for subunits that form a highly NO-sensitive sGC (Gycα-99B and Gycβ-100B) while three code for subunits that are weakly stimulated by NO (Gyc-88E, Gyc-89Da and Gyc-89Db) based on lysate assays with overexpressed protein (Morton et al. 2005). These weakly NO responsive sGCs have been termed atypical sGCs (Morton 2004), and have been identified based on sequence analysis in organisms ranging from *Caenorhabditis elegans* (Gcy31-Gcy37) to *Oncorhynchus mykiss* to *Manduca sexta* (MsGC-β3).

With the discovery that a distal pocket tyrosine is important for stabilizing O_2-binding in *Tt* H-NOX, the sequences of these predicted sGCs were examined to determine if they encode a tyrosine that aligns with *Tt* H-NOX Y140. Indeed, several of these atypical sGCs contain a tyrosine that is predicted to be in their heme distal pocket, which would suggest they bind oxygen. Experiments in mammalian cells did suggest that the *D. melanogaster* sGCs (Gyc-88E, Gyc-89Da and Gyc-89Db) were responsive to O_2 (Morton 2004), but this work did not directly examine ligand binding. This was only recently accomplished with the characterization of the purified Gyc-88E homodimer (Huang et al. 2007) which is the first and only report to show a Fe^{II}-O_2 complex in a guanylate cyclase. As expected, Gyc-88E also binds NO and CO. Interestingly, Gyc-88E is inhibited 2–3-fold by the binding of NO, CO and O_2, a property which is quite distinct from the ligand induced activation of the sGC α1β1 heterodimer.

There is a contradiction to this current proposal on ligand discrimination in sGC. The Gcy-35 H-NOX domain from *C. elegans* does bind O_2, but does not encode a tyrosine that aligns with *Tt* H-NOX Y140 based on multiple sequence alignments or a homology model generated using the *Tt* H-NOX structure. However, there is no crystal structure of this protein and it remains possible that an analogous hydrogen bond donor in the distal heme pocket stabilizes O_2 binding. Importantly, deletion of *gcy-35* disrupts cGMP-dependent behavioral responses to hyperoxia in worms, suggesting the protein functions as an O_2 sensor *in vivo* (Gray et al. 2004). Thus, although significant progress has been made in our understanding of ligand discrimination in sGC there remain some unknown variables that may contribute to the specificity of these heme proteins.

7 Conclusions

Although several biochemical reports have emerged over the past several years about sGC structure and function, the mechanism of sGC activation by NO remains unresolved. Clearly, the sGC response to NO is very complicated, and involves both NO binding to the heme and a second NO binding site. Furthermore, sGC is regulated by allosteric interactions with ATP and GTP. There are likely to be other important regulatory factors, perhaps including *S*-nitrosation. Despite the complexity of sGC, the H-NOX protein family has illuminated many important structural features of the sGC heme binding domain, and this family is likely to continue to shed light on the complicated activation and deactivation pathways.

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Genetic Mouse Models of the NO Receptor 'Soluble' Guanylyl Cyclases

Evanthia Mergia, Doris Koesling, and Andreas Friebe

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Abstract The NO/cGMP signalling cascade has an important role in smooth muscle relaxation, inhibition of platelet aggregation and neuronal transmission. Although the function of the main NO receptor GC (NO-GC) is well established, the particular tasks of the NO receptor isoforms (NO-GC1 and NO-GC2) are unclear and NO targets other than NO-GC have been postulated. Mice deficient in either NO receptor isoform or with a complete lack of NO-GC are now available and allow new insights in NO/cGMP signalling. The first reports about the KO strains show that, outside the neuronal system, the NO-GC isoforms can substitute for each other, and that amazingly low cGMP increases are sufficient to induce smooth muscle relaxation. In the neuronal system, however, the NO-GC isoforms obviously serve distinct functions as both isoforms are required for long term potentiation. Analysis of the complete NO-GC KO provides evidence that the vasorelaxing and platelet-inhibiting effects of NO are solely mediated by NO-GC. Thus, NO-GC appears to be the only NO receptor in these two systems.

1 Introduction

Shortly after the discovery of cAMP, cGMP was detected in many cells and tissues throughout the body (Schultz et al. 1969). In contrast to the formation of cAMP which was restricted to plasma membranes, cGMP-forming activity was found in

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particulate and cytosolic cell fractions (Chrisman et al. 1975). Although in the early days of guanylyl cyclase (GC) research, considerable effort was made regarding the characterization and isolation of the two enzyme forms, major progress was not achieved until the identification of the activators of the GC, i.e. nitric oxide for the soluble enzyme (Palmer et al. 1987) and atrial natriuretic peptide for the membrane forms (Kuno et al. 1986). The membrane-bound GCs and their respective activators are discussed in more detail in another chapter of this book. The discovery of nitric oxide as a signalling molecule received much attention and cumulated in awarding the Nobel price to Furchgott, Ignarro, and Murad. NO was shown to be produced by the NO synthases (NOS) with the neuronal (nNOS or NOSI) and endothelial NOS (eNOS or NOSIII) being of major importance for the production of NO as a signalling molecule. In contrast, the high amounts of NO produced by the inducible NOS (iNOS or NOSII) exert direct toxic effects. KO mice deficient for either one of the NOSs exist, the respective phenotypes will be compared with those of the NO-GC deficient mice below. The available research data strongly suggest that most of the effects of NO as a signalling molecule are mediated by the activation of NO-GC; however, speculations on cGMP-independent physiological effects of NO are numerous. Nevertheless, the cGMP-forming enzyme, GC, represents the only established NO receptor under physiological conditions identified so far and, therefore, will be termed NO receptor GC (NO-GC) in the following. Downstream in the NO/cGMP signalling pathway, the cGMP-dependent protein kinase (cGKI) mediates most of the cGMP effects. Studies with cGKI KO mice are described in more detail in another chapter of this book. Here, we will focus on NO-GC and after a short introduction of the NO receptor isoforms we will summarize the current knowledge obtained in genetic mouse models.

1.1 Isoforms of the NO Receptor Guanylyl Cyclase

GC has been first purified from lung, a tissue with a very high GC content (Gerzer et al. 1981a). The enzyme was shown to consist of two different subunits termed α and β with molecular weights of 73 and 70 kDa, respectively (Kamisaki et al. 1986) and to contain a prosthetic heme group which acts as the receptor for NO and is required to mediate the NO stimulation (Gerzer et al. 1981a; Gerzer et al. 1981b). The precise mechanism of NO activation is covered elsewhere in the book. With peptide sequences derived form the purified protein, cDNAs coding for the β subunit and α subunit have been cloned and sequenced (for review see Koesling et al. 1991 and references therein). Only coexpression of both subunits ($\alpha\beta$) yielded a catalytically active, NO-stimulated enzyme. Homology screening yielded cDNAs coding for two further subunits, α_2 and β_2, which in theory could give rise to four isoforms of the NO receptor GC. However, some evidence suggests that the β_2 cDNA may represent a pseudogene. In native tissues, the β_2 subunit has never been detected on the protein level and the mRNA content determined by quantitative PCR was extremely low (Mergia et al. 2003). Coexpression of the β_2 subunit with any other subunit did

not yield a catalytically active enzyme (own observation). In addition, a mutation found in the human β_2 gene causes a frame shift and impairs expression of the β_2 at least in humans (Behrends and Vehse 2000).

In contrast to the β_2 subunit, the α_2 subunit is able to form a catalytically active enzyme with the β_1 subunit. In precipitation experiments from placenta, the β_1 subunit was identified "in vivo" as the dimerizing partner of the α_2 subunit (Russwurm et al. 1998). Thus, two isoforms of the NO receptor GC exist, the heterodimers $\alpha_2\beta_1$ and $\alpha_1\beta_1$. As the isoforms share the common β_1 subunit but differ in their α subunit, they will be termed NO-GC1 and NO-GC2 in the following (Fig. 1). Extensive

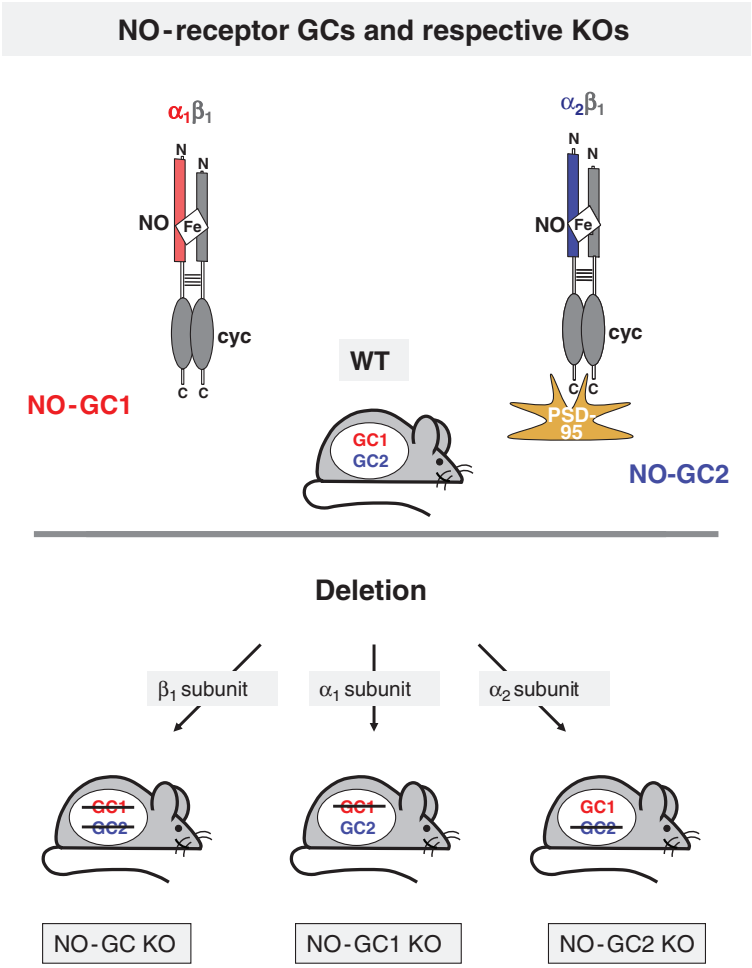

Fig. 1 Schematic illustration of the NO receptor GCs and the respective KOs. Shown is the subunit composition of the NO receptor GC isoforms ($\alpha_1\beta_1$, $\alpha_2\beta_1$) and the consequences following subunit deletion in the respective KO mice. For further explanation see text. GC, guanylyl cyclase; KO, knock out; cyc, cyclase catalytic domain

biochemical and kinetic analysis of both NO-GC1 and NO-GC2 did not reveal any significant differences especially regarding NO sensitivity, thus, the enzymatic regulation of the isoforms appears to be similar. Structurally, NO-GC2 was shown to be able to interact with adapter domains (PDZ domains) via its α_2 C-terminal peptide and a special intracellular localisation might represent the special feature of this isoform (Russwurm et al. 2001). Studies on the expression revealed a ubiquitous distribution of NO-GC1 and a more limited occurrence of NO-GC2 (Mergia et al. 2003). The highest expression of NO-GC2 is found in brain where both isoforms are present in similar amounts. In sum, the physiological significance of two similarly regulated NO-GCs is still unclear.

1.2 Genetic Mouse Models

Knock-out models offer the possibility to learn about a protein's function in its natural environment by comparing WT mice with mice deficient for this single protein. Mice lines deficient for each NO-GC subunit (α_1, α_2, β_1) have been generated (Mergia et al. 2006; Buys et al. 2008; Friebe et al. 2007). The knock-out of the β_1 subunit results in a complete loss of both NO-GC receptors whereas knock-out of either α subunit causes deletion of the respective NO-GC isoform (see Fig. 1). Accordingly, the NO-GC KO will reveal information about NO targets other than NO-GC, and the NO-GC1 and NO-GC2 KO mice will hopefully help to clarify the particular functions of the isoforms. All KO strains were generated with the Cre/loxP system. For the NO-GC1 two different KO strains exist; in addition, a mice strain with an NO receptor GC insensitive to NO but with retained non-stimulated cGMP-forming activity has been generated (preliminary report: Thoonen et al. 2007).

1.2.1 Phenotypes of NO-GC-Deficient Mice

The deletion of exon 10 of the β_1 subunit resulted in the complete loss of the β_1 protein; surprisingly, the α subunits could be not be detected on the protein level either (Friebe et al. 2007). In line with these findings, NO-induced cGMP synthesis was detected neither in lungs nor in brain nor in other GC-rich tissues/cells like aorta and platelets. Thus, deletion of the β_1 subunit leads to a complete loss of NO-GC.

The complete lack of NO receptor GC resulted in a drastic reduced life expectancy: 80% of homozygous KO mice died within the first 2 days. However, these mice are born at Mendelian ratio as 25% KO fetuses were detected when performing caesarean section. However, animals which survived the first two days lived for at least 18 days and practically all died within the following 2 weeks (90%). As weaning and thus a change in diet takes place around day 18, gastrointestinal complications, e.g. dysmotility of the gastrointestinal tract was considered as the most likely cause of death. This assumption was supported by an extremely prolonged

total gut transit time indicative of functional constipation and perforations of various parts of the gut found in post mortem analysis. In accordance with these symptoms, NO-GC KO mice had a grossly enlarged and dilated caecum which, in most cases, was displaced from its normal position. In mice lacking neuronal NOS (Gyurko et al. 2002), the lethal consequences of GI dysmotility could be avoided by feeding a standard liquid rodent diet; this was not the case in NO-GC KO mice. Yet, by feeding a low-residue diet, the mice were rescued indicating an impairment in the intestinal fiber transport. Taken together, the overall gastrointestinal phenotype emphasizes the prominent role of NO-GC and thus cGMP in the regulation of intestinal smooth muscle tone.

Comparing the gastrointestinal phenotype of the complete NO-GC KO with those of mice lacking cGKIs or NOSs reveals different degrees of overlap. Closest in phenotype are mice lacking cGKI which also show a reduced life span (less severe, as 50% die before 5–6 weeks of age) and very similar disturbances in the gastrointestinal tract (Pfeifer et al. 1998; Ny et al. 2000). There are two different KO strains for nNOS. In the first strain, exon 2 containing the start codon has been deleted. Yet, a splice variant, nNOSβ which lacks exon 2 has been shown to be synthesized and to be catalytically active in nNOS-KO mice (Eliasson et al. 1997; Hurt et al. 2006). This may be the reason for the comparatively mild gastrointestinal phenotype with enlargement of the stomach, hypertrophy of the pyloric sphincter and delayed gastric emptying (Huang et al. 1993). A second nNOS-KO line has been generated by the deletion of exon 6 (Gyurko et al. 2002). However, besides pyloric stenosis, a drastic gastrointestinal phenotype of the mice has not been reported.

The most interesting comparison can be made between GC-KO mice and a triple-KO strain in which seemingly all three NOS isoforms have been deleted (Morishita et al. 2005). The triple NOS-KO mice show a reduction in life expectancy with an 80% survival rate over 4–5 months which, surprisingly, is far less pronounced than in NO-GC-KO mice. It has to be noted though, that this "triple-KO" was derived from the original nNOS-KO generated by Huang et al. which still contains the nNOSβ splice variant. Thus, it is possible that the phenotype of these mice is still influenced by the residual NOS which is expressed in brain, penis and also in many parts of the GI tract (Saur et al. 2002). In sum, the further comparison of phenotypical similarities/differences between the NO-GC-KO mice and those lacking other members of the NO/cGMP cascade will help to improve the knowledge on possible diverse and synergistic functions of these signaling molecules.

1.2.2 Characteristics of NO-GC1- and NO-GC2-Deficient Mice

In contrast to the complete NO-GC KO, homozygous NO-GC1- and NO-GC2-KO mice were born at normal Mendelian ratio and did not show apparent alterations or a reduced life expectancy. On the protein level, removal of exon 4 of either α subunit led to a loss of the α subunit and the dimerizing β_1 subunit showing that one of the GC subunits is not stable without its dimerizing partner (Mergia et al. 2006). Vice versa, deletion of the β_1 subunit resulted in a loss of the α subunits as observed in

the NO-GC KO (see above). During the preparation of the current manuscript, the generation of another NO-GC1-KO strain was published (Buys et al. 2008). In these KOs, exon 6 of the α_1 subunit (corresponding to exon 5 in the numbering used by Mergia et al.) coding for a conserved portion of the catalytic domain was deleted. As the deletion did not cause a frameshift, a mutant α_1 subunit which was shown to be catalytically inactive upon coexpression with the β_1 subunit in insect cells is expressed in the respective NO-GC1 KO strain. Importantly, in all KO mice the loss of one NO-receptor GC was not compensated by up-regulation of the remaining one. Hence, cGMP-forming activity in either KO strain stands for the residual non-deleted NO receptor GC isoform (NO-GC1 or NO-GC2). The respective measurements were in good accordance with the tissue distribution analysis performed before (Mergia et al. 2003) and revealed comparatively high levels of the NO-GC2 in brain where the amount of this isoform equalled the amount of NO-GC1. The NO-GC1 was the only NO receptor isoform in platelets and the dominant isoform (>90%) in lung and aorta. As the NO-GC1 and NO-GC2 KO lines show normal life expectancy, the amounts of cGMP formed by either of the GC isoforms is apparently sufficient to prevent the high mortality found for the complete NO-GC KO (see above) and for the KO mice of the cGKI (Pfeifer et al. 1998). In the following, results obtained with the NO-GC and NO-GC1 KO mice in cGMP relevant systems will be introduced and discussed. As the NO-GC2 KO was indistinguishable from WT mice except for the neuronal system, the results will not be mentioned separately.

1.2.3 Smooth Muscle Tone

Role of NO-GC Isoforms in Vascular Smooth Muscle

Although the important role of the NO/cGMP cascade in smooth muscle relaxation is well established and the NO-GC1 has been identified as the major NO receptor isoform, the distribution and function of the NO-GC isoforms in vasculature needs to be determined. Analysis of the NO-GC receptor isoform content in aorta derived from the NO-stimulated cGMP-forming activities yielded approximately 94% and 6% for NO-GC1 and NO-GC2, respectively (Mergia et al. 2006). The NO-GC2 content in the arteria femoralis appears to be higher as judged by the cGMP-forming activity determined in the cytosolic fraction of femoral rings, which for unknown reasons, were determined only in the absence of NO and in the presence of the NO-sensitizer BAY 41–2272 (Nimmegeers et al. 2007a). Nevertheless, the NO-GC2 isoform has a profound functional impact on vascular tone as despite the only marginal NO-GC2 content, the NO-GC1 deficient rings completely relaxed in response to NO, although higher NO concentrations (fivefold higher EC50) were required (Mergia et al. 2006). These findings demonstrate an unpredicted functional role of NO-GC2 in vascular relaxation as the cGMP formed by this isoform is sufficient to cause relaxation. As expected, the NO-induced relaxation of NO-GC1-deficient rings was completely abolished in the presence of the inhibitor of NO-GC, ODQ, ensuring

that the retained NO-induced relaxation in the NO-GC1-deficient rings was caused by NO-GC2 and not by other NO effector molecules (Garthwaite et al. 1995).

In addition to the retained sensitivity towards NO, the NO-GC1 deficient-rings showed a preserved endothelium-dependent relaxation as they responded with a 50% relaxation to a carbachol concentration that evoked a complete relaxation of WT rings. Obviously, the cGMP increase elicited by endogenously produced NO is sufficient for a substantial relaxation in the NO-GC1-deficient rings; thus, small increases in cGMP exert a profound effect on vascular tone. Analysis of smooth muscle relaxation in aortic and femoral segments from another NO-GC1 KO strain yielded more or less similar results (Nimmegeers et al. 2007a, b). In this mice strain, sex-dependent hypertension with male but not female animals developing elevated blood pressure have been reported. In neither of the smooth muscle experiments gender specific differences were detected.

In contrast to the cGMP content in WT aortic rings which increased about ten-fold, cGMP levels in NO-GC1-deficient rings did not show a significant increase upon addition of NO. Similar results i.e. lack of NO-induced cGMP increases in the NO-GC1 KO aorta were reported by Nimmegeers et al. These results show that the cGMP formed by the residual NO-GC2 is not detectable and strongly suggest that local cGMP increases are responsible for the induction of the biological response. Taken together, the retained relaxation in the NO-GC1-deficient rings shows that the loss of the majority of the cGMP-forming NO receptor (94%) does not impair the biological response to but decreases the potency of NO. This opinion is not completely shared by Nimmegeers et al., who speculated about NO-GC-independent effects of NO in the NO-GC1 KO. This is puzzling as the retained relaxation in the NO-GC1-KO is sensitive to ODQ when used at a sufficiently high concentration. However, the argument is settled by the total loss of NO-induced relaxation in the complete NO-GC KO which clearly speaks against GC-independent effect of NO at least in aortic smooth muscle relaxation.

NO-GC1-deficient mice showed an increased sensitivity towards the activator of the membrane-bound GC-A, ANP, which can be interpreted as compensatory counter regulation. An alteration of cGMP-degrading phosphodiesterase content has been suggested to be able to compensate for high or low cGMP-forming activities (Kim et al. 2001, 2007). However, PDE activities measured in the aorta homogenate did not differ between KO and WT, therefore a reduced PDE expression as the underlying mechanism for the increased ANP sensitivity was ruled out. In addition to the increased sensitivity to ANP, the response to the direct activator of the cGKI, 8-pCPT-cGMP, was also increased in the NO-GC1-deficient aortic rings. The latter result was not confirmed by Nimmegeers et al., in their study relaxation induced by 8-pCPT-cGMP did not differ between WT and NO-GC1-KO.

Complete NO-GC KO in Smooth Muscle Relaxation

While the importance of NO for smooth muscle relaxation is beyond doubt, it is still unclear whether NO induces relaxation of vascular smooth muscle solely via NO-GC and/or via alternative mechanisms. Smooth muscle relaxation in NO-GC

KO aortic rings by various NO donors (GSNO, DEA-NO, Proli-NO, Angeli salt, SNP) was completely absent even at concentrations 3 orders of magnitude above those fully relaxing WT aorta. In line with these data, carbachol did not induce a relaxing response in the KO animals (Friebe et al. 2007). Yet, in the NO-GC-KO mice the signaling cascade downstream of GC functioned normally as relaxation induced by stimulator of cGKI 8-pCPT-cGMP was indistinguishable from that seen in WT mice. Thus, in the aorta, the relaxing effect of NO is solely conveyed by NO-GC.

The results are in good agreement with the abrogated endothelium-induced relaxation in the cGKI (Pfeifer et al. 1998) and eNOS KO mice (Huang et al. 1995; Chataigneau et al. 1999).

Smooth Muscle Relaxation in Corpus Cavernosum of NO-GC1 KO Mice

Smooth muscle relaxation is the key event in blood filling of the corporal tissue and restriction of venous outflow being required for penile erection (Andersson and Wagner 1995). The important role of NO/cGMP is underlined by the efficiency of the phosphodiesterase 5 inhibitor Sildenafil as today's most successful therapy for the treatment of erectile dysfunction. Both, NO produced by nNOS in non-adrenergic and non-cholinergic (NANC) nerves and NO produced by the eNOS are considered to be of importance in this physiological event (Musicki and Burnett 2006). While smooth muscle relaxation of NO-GC1 KO corpus cavernosum in response to acetylcholine, bradykinin and electric field stimulation (EFS) was almost completely lost, the NO releasing compound SNP led to substantial relaxation, the remaining relaxation being ODQ sensitive (Nimmegeers et al. 2007a, b). These results are reminiscent of those found in the vasculature indicating a role NO-GC2 in corporal smooth muscle relaxation obvious in the NO-GC1 KO mice.

1.2.4 Blood Pressure

The importance of NO/cGMP signalling for the maintenance of blood pressure is well established (Rees et al. 1989). Accordingly, the complete NO-GC KO mice showed a pronounced elevation of systolic blood pressure (26 mmHg) as determined with a non-invasive tail-cuff system. In contrast, systolic blood pressure of NO-GC1 KO mice, in which more than 90% of the aortic NO-GC is lacking, was only moderately elevated (7 mmHg) and did no show any sex difference (Mergia et al. 2006). Obviously, only a fraction of normal NO-GC content in the vessel wall is sufficient to control blood pressure. For the other NO-GC1 KO strain (Buys et al. 2008), gender-dependent hypertension was reported with higher blood pressure for male than female mice (147 vs. 118 mmHg). Hypertension was age- (after 14 weeks) and testosterone-dependent as prevented by orchidectomy and treatment with an androgen receptor antagonist. The discrepancy in gender-dependent hypertension may very well be related to the genetic background of the animals as the NO-GC1 KOs

are either on a mixed 129/C57BL6 (Mergia et al. 2006) or 129/Swiss or pure 129 background (Buys et al. 2008). When NO-GC1 KO mice were challenged with the NO synthase inhibitor L-NAME, the increase in blood pressure was comparable to that seen in WT mice demonstrating that NO-induced cGMP synthesis, although being greatly reduced, still has an important impact on vascular tone. The data underline the important role of constitutive NO production in blood pressure regulation and is in good accordance with results found in the eNOS and cGKI KO mice (Van Vliet et al. 2003; Pfeifer et al. 1998). Vice versa, injection of NO donors decreased blood pressure in WT and NO-GC1 KO mice (Vermeersch et al. 2007; Buys et al. 2008) whereas the NO-sensitizer BAY 41–2272 failed to lower blood pressure in NO-GC1 KO.

In accordance with the assumption that the blood pressure effects of NO are entirely mediated by the NO receptor GCs, the blood pressure decrease in response to an NO donor was totally abolished in the complete NO-GC KO mice (Friebe et al. 2007).

1.2.5 Cardiac Phenotype of NO GC1 KO Mice

Buys et al. performed invasive hemodynamic catheter measurements and report increased cardiac contractility and arterial elastance and as well as impaired ventricular relaxation in both male and female NO-GC1 KOs suggesting that NO-GC1 modulates myocardial function.

1.2.6 NO-Induced Inhibition of Platelet Aggregation is Exclusively Mediated by NO-GC1

Similar to smooth muscle relaxation, NO has been postulated to mediate inhibition of platelet aggregation by cGMP, although cGMP-independent mechanisms have been proposed as well (Moncada et al. 1998; Wanstall et al. 2005; Crane et al. 2005). Analysis of NO-GC KO platelets revealed a complete lack of an inhibitory effect of NO on platelet aggregation induced by various agonists (collagen, thrombin, U46619). Even millimolar concentrations of Proli-NO and high concentrations of other NO donors (GSNO, DEA/NO, SNP) were unable to induce inhibition of aggregation. Similar to aortic tissue, the signaling cascade beyond NO-GC in KO platelets was still intact as the inhibition of collagen-induced aggregation by 8-pCPT-cGMP, a direct cGKI activator, was as in WT (Friebe et al. 2007).

Analysis of the NO-GC receptor isoforms revealed NO-GC2 to be completely absent and thus, NO-GC1 to be the only isoform in platelet. In accordance, platelets from NO-GC1-deficient mice featured the same characteristics as those from the complete NO-GC KO (Mergia et al. 2006).

In sum, these data suggest that in platelets NO signals solely via NO-GC1 at least with regards to inhibition of aggregation. The results are in good accordance with those from cGKI KO (Massberg et al. 1999) and IRAG KO mice (Antl et al. 2007).

1.2.7 Gastrointestinal System

As outlined already before, the NO/cGMP cascade has an important role for the motility in the gastrointestinal system. Lack of the NO receptor caused a severe gastrointestinal phenotype characterised by an enlarged caecum, an extremely increased whole gut transient time resulting in fatal gastrointestinal obstruction. Under a normal diet, mice died shortly after weaning. However, under a fiber-free diet, survival of KO mice was greatly improved. In contrast, the GC1-deficient KO mice did not show any functional gastrointestinal impairment and visible abnormalities (Vanneste et al. 2007). Thus, the drastic changes observed in the complete NO-GC KO can almost be completely compensated by the relatively small amounts of cGMP formed by the NO-GC2 isoform.

Within the gastrointestinal tract, NO is considered to be an important neurotransmitter of NANC neurons. NO synthesized by nNOS is released from the NANC nerve terminals and is thought to diffuse to gastrointestinal smooth muscle cells where it causes relaxation via stimulation of NO-GC. The role of NO-GC1 in nitrergic regulation of gastric motility was studied with the respective KO mice (Vanneste et al. 2007). As the NO-stimulated cGMP activity was not determined in the intestine of those mice, the actual NO-GC2 isoform content is unknown but can be estimated to be low (Mergia et al. 2003). Whereas nitrergic relaxation induced by EFS was abolished or greatly reduced in the NO-GC1 KO mice; relaxation induced by exogenous NO was decreased but still substantial with the remaining relaxation being ODQ-sensitive. Basal cGMP levels were reduced in the NO-GC1 KO mice but NO still induced a moderate ODQ-sensitive increase in cGMP levels (3-fold vs. 34-fold in WT). In sum, the results show that NO-GC1 plays an important role in gastric nitrergic relaxation in vitro, but obviously in vivo the cGMP formed by the NO-GC2 isoform can substitute sufficiently to avoid drastic consequences in NO-GC1-deficient KO mice.

1.2.8 Pulmonary Vasodilation

The NO/cGMP signalling cascade has been implicated to play a role in pulmonary vascular tone and vascular remodelling as the NO-GC content is comparatively high and inhalative NO selectively dilates pulmonary resistance vessels (Ichinose et al. 2004). A study evaluating pulmonary pressure and vascular remodelling in mice lacking the NO-GC1 has been published (Vermeersch et al. 2007). In this report, measurements of NO-stimulated cGMP-forming activity yielded less than 1.5% of WT activity in the NO-GC1 deficient lung suggesting even lower amounts of NO-GC2 in lung than the 7% reported by Mergia et al. in their NO-GC1 deficient mouse strain. The NO-GC1 KO mice did not show pulmonary arterial hypertension under normal conditions as determined by right ventricular systolic pressure, nor did the response to acute hypoxia differ from the one in WT. These results indicate that NO-GC1 does not have an essential role in pulmonary vascular tone under physiological conditions or in the acute vasoconstrictor response to hypoxia. However, the

reversal of hypoxia-induced increase in systolic pressure in the WT by NO did not occur in the NO-GC1 KO. Under prolonged hypoxia (3 weeks), the authors report an upregulation of the α_1 and β_1 subunit mRNA and protein levels in WT which is not paralleled by enhanced NO-GC activity. In addition, the increase in right ventricular pressure, right ventricular hypertrophy and remodelling of pulmonary vessels observed under chronic hypoxia was significantly greater in NO-GC1 KO mice. The results suggest that NO-GC1 serves to limit hypoxia-induced pulmonary vascular remodelling. Pulmonary hypertension was found in the NOS3 mice (Steudel et al. 1998), the lack of this alteration in the NO-GC1 KO can most likely be explained by the NO-GC2 in the NO-GC1 KO.

1.2.9 Synaptic Transmission

NO has been postulated to participate in certain types of synaptic plasticity such as long term potentiation, the use-dependent increase of transmission efficacy at synapses implicated in learning and memory (reviewed in Garthwaite and Boulton (1995) and Hawkins et al. (1998)). Initial reports already suggested NO as retrograde messenger synthesized in response to NMDA receptor activation in the postsynaptic neuron and causing long-lasting increases in the transmitter release in the presynaptic terminals (Garthwaite et al. 1988; O'Dell et al. 1991; Schuman and Madison 1991). Various experimental studies performed thereafter revealed that NO is not involved in all forms of LTP but only in certain areas under special circumstances (Son et al. 1998). Analysis of NOS KOs indicated an NO-dependent form of LTP in the stratum radiatum of the CA1 field of the hippocampus (Son et al. 1996; Wilson et al. 1997, 1999).

The role of the NO-GC isoforms in LTP was studied in the visual cortex as both NO-GC isoforms are expressed in the visual cortex as are both NO-producing enzymes, eNOS and nNOS (Haghikia et al. 2007). Moreover, the cGMP amount measured in NO-incubated slices of visual cortex was similar to that found in the hippocampal CA1 region indicating comparable amounts of NO-GC in both brain areas. LTP measured in WT cortical slices was completely prevented by the inhibitor of NO-GC, ODQ, indicating that NO/cGMP participates in synaptic plasticity in the visual cortex. Unexpectedly, LTP was absent in either one of the NO-GC isoform deficient mice but restorable by a cGMP analogue paired with a theta-burst stimulus. The successful reconstitution of LTP by exogenous cGMP in the KO strains is of major importance demonstrating normal processing of synaptic transmission in the NO-GC1 and NO-GC2 KOs. Our finding that both NO-GC isoforms play a role in LTP and cannot substitute for each other indicates that two distinct NO/cGMP pathways are required within LTP. Two spatially or temporally distinct effects of NO/cGMP have already been suggested. Based on the finding that LTP induced by exogenous NO paired with a short tetanus was blocked by inhibiting NOS, two NO/cGMP events were proposed to be required for the induction and maintenance of LTP (Bon and Garthwaite 2003). In a recent report, NO derived form eNOS and from nNOS were shown to be required for LTP (Hopper and Garthwaite 2006) which might explain the finding that LTP was found to be abolished only in the

double NOS KO (nNOS and eNOS; Son et al. 1996). In sum, the data suggest distinct roles of the NO-GC isoforms in synaptic transmission and hopefully the elucidation of their precise functions will improve our understanding of the molecular mechanisms underlying LTP and possibly plasticity in general.

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Function and Dysfunction of Mammalian Membrane Guanylyl Cyclase Receptors: Lessons from Genetic Mouse Models and Implications for Human Diseases

Michaela Kuhn

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Abstract Besides soluble guanylyl cyclase (GC), the receptor for NO, there are seven plasma membrane forms of guanylyl cyclase (GC) receptors, enzymes that synthesize the second-messenger cyclic GMP (cGMP). All membrane GCs (GC-A to GC-G) share a basic topology, which consists of an extracellular ligand binding domain, a short transmembrane region, and an intracellular domain that contains the catalytic (GC) region. Although the presence of the extracellular domain suggests that all these enzymes function as receptors, specific ligands have been identified

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for only four of them (GC-A through GC-D). GC-A mediates the endocrine effects of atrial and B-type natriuretic peptides regulating arterial blood pressure and volume homeostasis and also local antihypertrophic and antifibrotic actions in the heart. GC-B, the specific receptor for C-type natriuretic peptide, has a critical role in endochondral ossification. GC-C mediates the effects of guanylin and uroguanylin on intestinal electrolyte and water transport and epithelial cell growth and differentiation. GC-E and GC-F are colocalized within the same photoreceptor cells of the retina and have an important role in phototransduction. Finally, GC-D and GC-G appear to be pseudogenes in the human. In rodents, GC-D is exclusively expressed in the olfactory neuroepithelium, with chemosensory functions. GC-G is the last member of the membrane GC form to be identified. No other mammalian transmembrane GCs are predicted on the basis of gene sequence repositories. In contrast to the other orphan receptor GCs, GC-G has a broad tissue distribution in rodents, including the lung, intestine, kidney, skeletal muscle, and sperm, raising the possibility that there is another yet to be discovered family of cGMP-generating ligands. This chapter reviews the structure and functions of membrane GCs, with special focus on the insights gained to date from genetically modified mice and the role of alterations of these ligand/receptor systems in human diseases.

Abbreviations

GC Guanylyl cyclase
ANP Atrial natriuretic peptide
BNP B-type natriuretic peptide
CNP C-type natriuretic peptide
PDE Phosphodiesterase
PKG cGMP-dependent protein kinase
CNG Cyclic nucleotide-gated

1 Introduction: General Features of Membrane Guanylyl Cyclases

1.1 Protein Structure and Signaling

In the past 25 years, the purification, cloning, and expression of various forms of guanylyl cyclase (GC) have revealed that besides soluble GC, the receptor for NO, there are seven other mammalian plasma membrane enzymes that synthesize the second-messenger cGMP. Although partly homologous to soluble GC, the membrane GCs (GC-A to GC-G) share a unique topology that consists of an extracellular ligand binding domain, a short transmembrane region, and an intracellular domain that contains the catalytic (GC) region at its C-terminal end (Fig. 1). The presence

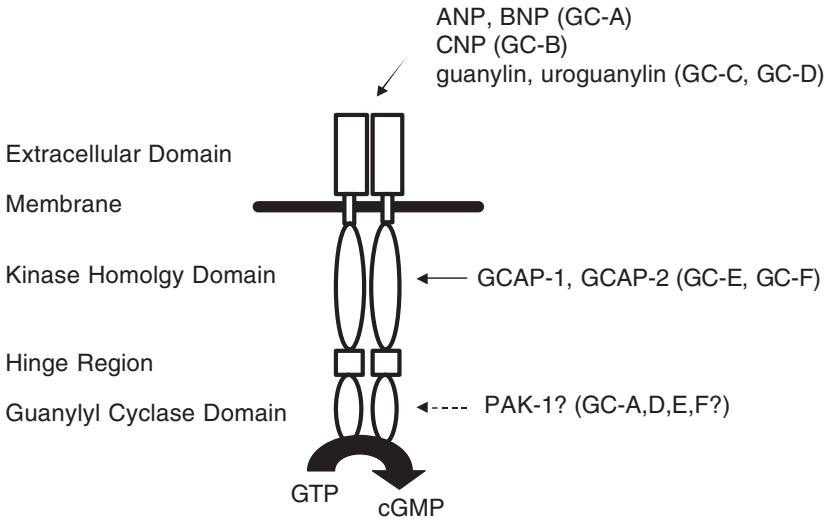

Fig. 1 Predicted shared basic topology and activators of membrane GC-A through GC-G. A single transmembrane segment separates an extracellular domain from intracellular protein kinase–like, hinge, and cyclase catalytic domains. All membrane forms appear to form homodimers or higher ordered structures

of the extracellular domain suggests that all these enzymes function as receptors for specific ligands, but such ligands have been identified for only four of them (GC-A to GC-D), with the remaining three forms (GC-E through GC-G) presumed as orphan receptors. In contrast to soluble GC activators, which are small gaseous molecules (NO and CO), activators of membrane GCs are peptides. Their ligands and most prominent tissue expression sites are depicted in Table 1. For clarity, the table also lists the different names which have been used in the literature to refer to the various membrane GCs. This review will use the nomenclature, which was applied by David L. Garbers, whose laboratory successively cloned each of them (University of Texas, Southwestern, Dallas TX) (Garbers and Lowe 1994).

The intracellular region of all membrane GCs consists of a juxtamembraneous protein kinase–homology domain (KHD), an amphipathic α-helical or hinge region, and a C-terminal cyclase catalytic domain (Fig. 1) (Chinkers and Garbers 1989; Wilson and Chinkers 1995). The function of the KHD is incompletely understood. Although it binds ATP and contains many residues conserved in the catalytic domain of protein kinases, kinase activity has not been detected. It modulates the enzyme activity of the C-terminal cyclase–homology catalytic domain (Chinkers and Garbers 1989; Koller et al. 1992) and may act as a docking site for the direct association of membrane GCs with other proteins, such as the intracellular Ca^{2+}-binding GC-activating proteins, GCAP-1 and GCAP-2 that regulate retinal GC-E and GC-F function, respectively (Laura and Hurley 1998; Palczewski et al. 2004). The coiled-coil hinge region is involved in higher order oligomerization. Although the transmembrane GC receptors contain a single cyclase catalytic active

Table 1 Nomenclature, prominent regions of expression, ligands and main functions of the various mammalian membrane guanylyl cyclase receptors

Receptor	Nomenclature	Main tissue distribution	Extracel. ligands	Functions
GC-A	Natriuretic peptide receptor-A (NPR-A), NPR1	Vascular smooth muscle, endothelium, heart central and peripheral nervous system adrenals, spleen, kidney	ANP, BNP	Decrease in arterial blood pressure, Decrease in blood volume, Inhibition of cardiomyocyte growth, Inhibition of cardiac fibrosis.
GC-B	Natriuretic peptide receptor-B (NPR-B), NPR2	Vascular smooth muscle, endothelium, heart bone	CNP	Inhibition of cardiomyocyte growth, Vascular regeneration, Endochondral ossification.
GC-C*		Intestinal epithelium, kidney, regenerating liver	Heat-stable entero toxins, guanylin, uroguanylin	Increased intestinal electrolyte and water transport, Epithelial cell growth and differentiation, Diuresis, natriuresis?
GC-D		Olfactory neuroepithelium	guanylin, uroguanylin	pheromone detection? salt and water homeostasis? *Pseudogene in the human*
GC-E	Retinal GC1	Retina, pineal gland, olfactory bulb, cochlear nerve, organ of Corti	Orphan	Vision, survival of cones
GC-F	Retinal GC2	Retina	Orphan	Vision
GC-G		Skeletal muscle, lung, intestine, sperm, kidney	Orphan	Renal protection? *Pseudogene in the human*

*GC-C should not be confounded with NPR-C, the natriuretic peptide receptor C, a second specific receptor subtype for ANP, BNP and CNP. NPR-C is a "clearance" receptor that is devoid of guanylyl cyclase activity and which mediates the cellular internalization and degradation of NPs.

site per polypeptide chain, receptor dimerization is essential for the activation of the catalytic domain (Wilson and Chinkers 1995; Yang and Garbers 1997). The homodimeric structure of particulate GCs is opposed to the heterodimeric structure of soluble GC. Misono and colleagues solved the crystal structure of the ECD of GC-A with and without bound ANP (reviewed by Misono et al. 2005). Their studies suggest that an ANP-induced rotation of the juxtamembrane domains, transduced across the membrane, may reorient the intracellular domains such that the catalytic sites of the two GC domains are brought to an optimal proximity and orientation, thereby giving rise to the synthesis of cGMP. This model of transmembrane signal transduction might be valid for all ligand-stimulated membrane GCs. Presumably, all effects of the membrane GCs are mediated by the synthesis of cGMP as an intracellular signaling molecule, which then modulates the activity of specific target

proteins, such as phosphodiesterases (PDEs 2, 3 and 5), ion channels, and cGMP-dependent protein kinases (PKG I and II), and thereby modifies cellular functions (see specific chapters in this series of reviews).

As just mentioned, the activity of the retinal transmembrane GCs, GC-E and GC-F, is modulated by retinal-specific intracellular proteins, named GCAPs. Until recently, such direct "ligand-independent" activation was not known for the other mammalian membrane GCs. Intriguingly, studies by Guo and colleagues (2007) in overexpressing cells showed that the small GTPase, Rac can use its effector PAK (p21-activated kinase) to directly activate transmembrane GCs (GCs A, D, E, and F), thereby leading to a ligand-independent increase in cellular cGMP levels. Apparently, PAK1 activates membrane GCs by direct contact with the cyclase domain to induce a conformational change. The authors showed that this new Rac/PAK/GC/cGMP pathway is involved in platelet-derived growth factor (PDGF)-induced fibroblast migration in vitro (Guo et al. 2007). Whether these findings provide a general mechanism for diverse endogenous receptors to crossactivate membrane GCs in vivo remains an attractive hypothesis. Also, at first sight, it seems difficult to reconcile these observations with other studies demonstrating a pronounced inhibitory effect of PDGF and other mitogens on both basal and ligand-dependent cGMP production by GC-B (see Sect. 1.2).

1.2 Posttranslational Modifications Regulate GC Responsiveness and Activity

Although studies to characterize the regulation of membrane GCs by posttranslational modifications have been mostly conducted with GC-A and GC-B, these may serve as prototypes for the other membrane forms. Hence, biochemical studies in GC-A–overexpressing cells showed that phosphorylation of GC-A within the KHD is essential for its activation process (Potter and Garbers 1992; Potter and Hunter 1998; Foster and Garbers 1998). In turn, desensitization and/or inactivation of GC-A in the presence of ANP involves dephosphorylation of the KHD (Potter and Garbers 1992). On these experiments, the groups of Garbers and Potter constructed a working model of the process of ANP- and BNP-dependent activation and deactivation of GC-A (reviewed by Potter and Hunter 2001). In the absence of NP, the receptor is highly phosphorylated on at least six amino acid residues located within the KHD, and its GC activity is repressed. On NP binding, a conformational change occurs that facilitates three subsequent events. First, the normal inhibitory effect that the KHD has on catalytic activity is relieved. Second, an increased dissociation rate decreases the affinity of the extracellular domain of GC-A for ANP and BNP (Jewett et al. 1993). Third, a conformational change in the KHD may expose the phosphorylated residues to a specific protein phosphatase. The resulting dephosphorylated receptor is unresponsive to further hormonal stimulation.

The responsiveness of GC-A and GC-B can be reduced not only by chronic exposure to NPs (homologous desensitization) but also by exposure to agents other

than NPs such as Angiotensin II and endothelin (GC-A) and mitogens such as PDGF, fibroblast growth factor, or serum (GC-B) (Haneda et al. 1991; Chrisman and Garbers 1999; Potter and Hunter 2001). Again, this heterologous desensitization of the GCs correlates with the phosphorylation state of the receptors. On the basis of these observations, it is conceivable that increased local concentrations of certain hormones and growth factors interfere with NP to GC-A/GC-B signaling under certain pathological conditions in vivo. Unfortunately, the specific protein kinase(s) and phosphatase(s) involved in the phosphorylation (sensitization) and dephosphorylation (homologous vs. heterologous desensitization) of GC-A and GC-B remain largely unknown. Both protein phosphatases 2A and 2C have been involved in homologous desensitization of GC-A (Bryan and Potter 2002). Further, it was shown that cAMP-dependent protein kinase (PKA) is somehow involved in homologous, but not heterologous, desensitization of GC-A in a leydig cell line (Müller et al. 2006). Overall, the specific proteins involved in the regulation of membrane GC receptors remain unidentified. It is also not known whether and how phosphorylation/dephosphorylation affects the activity of other membrane GCs.

Another common feature of all membrane GCs, with the exception of GC-F, is the glycosylation of residues located at the N-terminal end of the extracellular domain. The functional consequences remain controversial (Müller et al. 2002). In studies with cells overexpressing GC-A, GC-B, or GC-C, removal of carbohydrate residues with endoglycosidase prevented or reduced ligand binding, indicating that glycosylation plays a role in ligand binding (Lowe and Fendly 1992; Fenrick et al. 1997; Hasegawa et al. 1999). Others instead suggested that glycosylation functions in folding and/or transport of particulate GCs to the cell membrane (Miyagi et al. 2000).

1.3 Compartmentation of cGMP Produced by Membrane Vs. Soluble GCs

Many cells express both soluble (sGC) and particulate forms of GC but exhibit distinct responses to cGMP produced by either form. For instance, it was shown that regulation of cardiac β-adrenergic response by cGMP is specifically linked to NO – activated sGC. ANP stimulation achieved greater detectable increases in cGMP but did not modulate β-adrenergic responses (Takimoto et al. 2007). In neonatal rat cardiomyocytes and hepatocytes, ANP/GC-A, but not NO/soluble GC, stimulated the translocation of cGMP-dependent protein kinase I (PKG I) to the plasma membrane (Airhart et al. 2003; Stratton et al. 2008). These and many other studies raised the question, whether and how cGMP production and signaling is compartmentalized within cells. Castro and colleagues monitored subsarcolemmal cGMP signals in adult rat cardiomyocytes, by expression of a cyclic nucleotide-gated (CNG) channel and recording of the associated cGMP-gated current (Castro et al. 2006). The results indicate that in rat cardiomyocytes (1) cGMP produced by GC-A is readily accessible at the plasma membrane, whereas cGMP produced by sGC is not; and (2) PDE 5 controls the soluble but not the particulate pool, whereas the latter is

under exclusive control of PDE2. More direct methods have been recently developed to monitor cGMP changes using fluorescent probes and imaging microscopy. As a very elegant example, FlincGs (fluorescent indicators of cGMP, composed of cpEGFP, N-terminally fused to regulatory domain fragments of PKG) allowed the direct examination of local cGMP dynamics in real-time (Nausch et al. 2008). In vascular smooth muscle cells, this revealed transient global cGMP elevations in response to NO and sustained submembrane cGMP elevations in response to ANP. Taken together, these studies demonstrate a differential spatio-temporal intracellular distribution and action of cGMP produced by particulate vs. soluble GCs (reviewed by Fischmeister et al. 2006). These studies also suggested that cGMP-specific PDEs contribute to maintain these local patterns of $[cGMP]_i$.

2 GC-A Is the Receptor for Two Cardiac Peptides, ANP and BNP

In 1981, de Bold and colleagues published a landmark study showing that rat atrial extracts contain a potent diuretic and natriuretic factor, thereby establishing for the first time the connection between the heart and the kidney (de Bold et al. 1981). This observation led to the isolation of ANP from cardiac tissue (Kangawa and Matsuo 1984). Subsequently, two other peptides possessing a common core structure were recognized and named in alphabetical order, BNP and CNP (Sudoh et al. 1988, 1990). Because ANP possessed strong natriuretic properties, all three molecules have been referred to as NPs, even though they possess other activity or may never function to regulate sodium excretion (especially CNP; see Sect. 3). ANP and BNP are cardiac hormones that are produced mainly in the atrium and ventricle, respectively (de Bold et al. 2001). They are released into the bloodstream, activate the GC-A receptor, which is expressed in a variety of tissues, and thereby modulate blood pressure/volume. Outside the heart, low levels of ANP-mRNA or proANP-like immunoreactivity have been detected in aortic arch cells, lung, brain, adrenals, kidney, gastrointestinal tract, thymus, choroidea, and ciliary bodies, probably with autocrine/paracrine functions (reviewed by Ruskoaho 1992). In the kidney, alternative processing of the ANP precursor generates a 32–amino acid peptide called urodilatin, which may be important for the local regulation of renal sodium and water handling via GC-A (Schulz-Knappe et al. 1988).

The important role of the ANP/GC-A system in the physiological regulation of arterial blood pressure/volume has been emphasized in different genetic mouse models (Table 2). Targeted deletion of the peptide (ANP −/−) or its receptor (GC-A −/−), leads to severe chronic arterial hypertension, hypervolemia, and cardiac hypertrophy (Table 2) (Lopez et al. 1995; John et al. 1996; Oliver et al. 1997; Skryabin et al. 2004). In contrast, overexpression of ANP or GC-A elicits a “dose-dependent” fall in arterial blood pressure (Steinhelper et al. 1990; Oliver et al. 1998). Intriguingly, although BNP and ANP appear to signal through the same receptor, mice without BNP exhibit a different phenotype than do ANP-deficient mice. Whereas,

Table 2 Mouse models resulting from genetic alterations of natriuretic peptides or their receptors

Genetic alteration	Phenotype	References
ANP deletion	Salt-sensitive hypertension, pulmonary hypertension	John 1996
BNP deletion	Cardiac fibrosis	Tamura 2000
CNP deletion	Altered endochrondral ossification, dwarfism, early death	Chusho 2001
Guanylin deletion	Increased proliferation of colonic epithelia, enlarged crypts	Steinbrecher 2002
Uroguanylin deletion	Increased blood pressure, impaired natriuretic response to enteral NaCl load	Lorenz 2003
GC-A deletion	Salt-resistant hypertension, hypervolemia, cardiac hypertrophy and fibrosis	Lopez 1995; Oliver 1997; Skryabin 2004
Smooth muscle cell-GC-A deletion	Arterial normotension, hypertensive reaction to acute vascular volume expansion	Holtwick 2002b
Endothelial – GC-A deletion	Mild hypervolemia and hypertension	Sabrane 2005
Cardiomyocyte-GC-A deletion	Arterial hypotension, cardiac hypertrophy, impaired diastolic relaxation	Holtwick 2003
GC-B deletion	Dwarfism, seizure attacks, infertility, misguidance of sensory axons	Tamura 2004; Schmidt 2007
GC-C deletion	Resistance to heat-stable enterotoxins, elongated colonic crypts	Schulz 1997; Li 2007
GC-D deletion	Abolished calcium-responses of olfactory epithelium to (uro)guanylin	Leinders-Zufall 2007
GC-E deletion	Selective dystrophy of retinal cones	Yang 1999
GC-F deletion	No phenotype	Baehr 2007
GC-E + F deletion	Cone/rod dystrophy	Baehr 2007
GC-G deletion	Protection from renal ischemic damage	Lin 2008

BNP – deficient mice do not have hypertension or cardiac hypertrophy and are susceptible to cardiac fibrosis (Table 2) (Tamura et al. 2000). Thus, gene-deletion experiments suggest that ANP and BNP have distinct physiological roles. Under physiological conditions (in the absence of cardiac pressure or volume overload), the peripheral circulating plasma concentrations of BNP are much lower than the concentrations of ANP. Also, the affinity of BNP to bind GC-A is ~8-fold lower than that of ANP (Bennett et al. 1991). Accordingly, the potency for vasorelaxation is also markedly less (Van der Zander et al. 1999). Thus, it is possible that BNP (which is constitutively expressed in ventricular cardiomyocytes) mainly acts as a local paracrine antifibrotic factor within the heart (i.e., because fibroblasts express relatively high levels of both GC-A and GC-B).

2.1 Endocrine Actions of the ANP/GC-A System Cooperate in the Regulation of Blood Pressure and Blood Volume

The main known GC-A-mediated hypovolemic and hypotensive actions of ANP include the following: stimulation of renal function; vasodilatation; increased fluid efflux from the intravascular to the lymphatic system within the spleen; inhibition of the renin-angiotensin-aldosteron (RAA) system by direct actions on juxtaglomerular cells and the adrenal glomerulosa; and central nervous effects that decrease salt appetite and water drinking (reviewed in Brenner et al. 1990; Kuhn 2003). The contribution of these different GC-A-mediated actions of ANP to blood pressure and volume homeostasis is controversial. For instance, although ANP clearly counteracts the RAA system at the level of its expression and function (Johnston et al. 1989), renin and aldosterone levels are not increased in adult GC-A −/− mice (Shi et al. 2001; Holtwick et al. 2002a) and despite this, these mice are clearly hypertensive. Thus, the chronic reduction in blood pressure/volume by ANP might be mediated mainly by other actions of the hormone, such as the stimulation of diuresis and natriuresis. Within the vascular system, the GC-A receptor is densely expressed both in smooth muscle and in endothelial cells. To dissect these vascular actions of ANP in vivo, we inactivated the GC-A gene selectively either in smooth muscle cells (SMC) or in endothelia (EC), using Cre-lox technology (see Table 2). Remarkably, smooth muscle-restricted deletion of GC-A in mice completely abolished the direct vasodilating effect of ANP but did not affect resting arterial blood pressure (Holtwick et al. 2002b). In contrast, endothelium-restricted GC-A deletion, preserved ANP vasodilatation but caused hypertension (Sabrane et al. 2005). Physiological and Doppler echocardiography studies showed that EC GC-A KO mice had chronic hypervolemia, with the total plasma volume being expanded by 11–13%. By comparison, mice with global, systemic GC-A deletion, plasma volume is chronically increased by ∼30% (Skryabin et al. 2004). This demonstrates that the endothelial GC-A receptor mediates in a significant part the long-term balance of intravascular volume by ANP. Our recent vital microscopy studies in mice with dorsal skinfold chambers showed that ANP increases the microvascular extravasation of fluorescently labelled albumin (unpublished observations). This effect is totally abolished in EC GC-A KO mice. We conclude that ANP enhances microvascular macromolecule permeability and thereby shifts the balance of hydrostatic and colloid osmotic forces across capillary walls in favor of moving protein-free fluid from the plasma into interstitial pools. This ultimately decreases intravascular volume and blood pressure. Modulation of endothelial permeability via GC-A may in fact represent one of the physiologically most important actions of ANP.

2.2 Local Cardiac Functions of the ANP/GC-A System Counteract Cardiac Hypertrophy and Fibrosis

During chronic hemodynamic overload, the expression levels of ANP and especially, BNP in the cardiac ventricles significantly increase (de Bold et al. 2001).

In vitro studies suggested that NPs in this situation may act not only as circulating endocrine factors but also as local antihypertrophic (ANP) and antifibrotic (BNP) cardiac factors (Calderone et al. 1998). To test whether NPs locally modulate cardiomyocyte growth in vivo, we generated mice with selective deletion of GC-A in ventricular cardiomyocytes through Cre-Lox recombination (Holtwick et al. 2003). Already under resting conditions, these mice exhibit mild cardiomyocyte hypertrophy and elevation of known hypertrophy-marker genes. In addition, they respond to pressure overload (transverse aortic constriction) or systemic Angiotensin II administration with exacerbated cardiac hypertrophy and fibrosis (Holtwick et al. 2003; Kilic et al. 2007). In a latter study, cardiac GC-A was inhibited through the overexpression of a dominant negative form of GC-A in transgenic animals (Patel et al. 2005). This again resulted in a modest accentuation of the hypertrophic response to pressure overload, together with a dramatically increased cardiac fibrotic response. Importantly, as mentioned above, in the absence of any increase in arterial pressure, the cardiac phenotype of mice lacking the BNP gene was fibrosis (Tamura et al. 2000). Extending these observations, two reports have demonstrated that, in cultured cardiac fibroblasts, BNP stimulates selective matrix metalloproteinases that degrade collagen and suppress profibrotic extracellular matrix genes activated by transforming growth factor-β (TGF-β) (Tsuruda et al. 2002; Kapoun et al. 2004). These studies, taken together, support an important autocrine/paracrine role for the ANP-BNP-GC-A signaling pathway, which moderates myocyte growth and controls extracellular matrix production.

In summary, these observations from various genetic mouse models with global or conditional cell-restricted deletion of GC-A emphasize the importance of the NP/GC-A/cGMP system in the endocrine regulation of arterial blood pressure and blood volume and also its local actions on cardiac remodeling. In addition to its role within the cardiovascular system, recent reports have indicated that GC-A might be critical in the regulation of other completely different physiological processes, such as cellular growth in the brain and kidney (Brenner et al. 1990), angiogenesis (Kook et al. 2003), liver regeneration (Carini et al. 2003), or even lipolysis and lipid mobilization in adipose tissue (Lafontan et al. 2005).

2.3 Relevance of Alterations in the ANP/GC-A System to Cardiovascular Diseases

Quantitative alterations in gene expression governed by polymorphisms in noncoding sequences contribute to the genetic susceptibility to complex diseases, such as essential hypertension (EH) and cardiac hypertrophy. Gene polymorphisms of ANP or GC-A have been associated with EH (Rutledge et al. 1995; Beige et al. 1997) and with increased left ventricular mass in EH (Rubattu et al. 2006). Knowles et al. (2003) identified 10 polymorphic sites in the noncoding sequence of GC-A and, by transient expression analysis, demonstrated that they can alter GC-A expression as much as 2-fold.

Apart from these genetic variations, functional alterations of ANP or GC-A might also be involved in cardiovascular diseases. Inappropriate ANP secretion was reported in salt-sensitive hypertensive black patients, manifesting a paradoxical decrease in ANP secretion under conditions of high salt intake (Campese et al. 1996). However, blunted vasodilating, diuretic/natriuretic and cGMP responses to exogenous ANP have been reported in EH (Bulut et al. 2003), in Cushing's disease (Sala et al. 2001) and in patients with cardiac hypertrophy or congestive heart failure (Burnett et al. 1986; Hirooka et al. 1990). The latter patients have elevated plasma levels of ANP and BNP, with these levels being highly related to the severity of the disease (Burnett et al. 1986). However, the responses to exogenous ANP or BNP are markedly attenuated, indicating a downregulation or impaired receptor or postreceptor responsiveness of GC-A. The processes of homologous and heterologous desensitization of GC-A might described in Sect 1.2 be involved.

3 The CNP/GC-B System Forms a Paracrine Axis to Regulate Cell Growth: Mutations in GC-B Cause a Severe Form of Dwarfism

GC-B (also referred to as NPR-B or NPR2) is the specific receptor for the third member of the "natriuretic peptide family", C-type natriuretic peptide (CNP). While ANP and BNP expression is found primarily in the heart, CNP expression is more diffuse. Expression has been described in vascular endothelial cells, growth plate cartilage, uterus, ovaries, brain, and other tissues (reviewed by Olney 2006a). CNP does not circulate in the blood in appreciable amounts and therefore may act locally. The GC-B receptor is also expressed in many different tissues, in particular in vascular endothelial and smooth muscle cells, regions of brain and bone, and at high density in fibroblasts (Olney 2006a). In pharmacological experiments, the potency of CNP for vasodilatation or reduction of blood pressure is much lower as compared to ANP (Wei et al. 1994; Lopez et al. 1997). Even more, ablation of the peptide or its receptor in mice does not result in arterial hypertension (Tamura et al. 2004), questionating a physiological role for CNP/GC-B in the regulation of vascular tone. In contrast, various studies suggested that CNP/GC-B signaling within the vascular wall might be involved in the local modulation of vascular regeneration. Intriguingly, CNP seems to stimulate proliferation of endothelial cells but to attenuate proliferation of smooth muscle cells via GC-B (Komatsu et al. 1996; Yamahara et al. 2003), suggesting a role in angiogenesis. Within the heart, locally produced CNP might inhibit cardiac fibroblast proliferation and myocyte hypertrophy through GC-B (Pagel-Langenickel et al. 2007).

The most critical functions of CNP/GC-B have been unmasked by the severe consequences of genetic dysfunctions of this ligand/receptor system. Ablation of CNP, GC-B or cGMP-dependent protein kinase II (PKG II) in mice, all resulted in severe dwarfism as a result of impaired endochondral ossification, the long bones and

vertebrae of these knockout mice measuring only 50–80% of wild type littermates (see Table 2) (Pfeifer et al. 1996; Chusho et al. 2001; Tamura et al. 2004). Mice heterozygous for the mutation are slightly, but significantly, shorter. Vice versa, transgenic mice overexpressing CNP are overgrown. These and other observations demonstrated that CNP/GC-B/PKG II is a local regulatory system in the bone, where it activates growth plate chondrocyte proliferation and differentiation. The distal intracellular pathways mediating these effects are largely unknown (Olney 2006a). In humans, homozygous GC-B mutations are the cause of acromesomelic dysplasia, Maroteaux type (AMDM), a severe form of autosomal recessive, short-limbed dwarfism with characteristic skeletal deformities (Bartels et al. 2004). These patients have few nonskeletal-related problems and are of normal intelligence. Heterozygous carriers of GC-B mutations also have reduced stature (Olney et al. 2006b). Remarkably, the studies by Warman's group suggest that approximately one in 30 individuals with idiopathic short stature is heterozygous carrier of GC-B mutations (Olney et al. 2006b).

In summary, disruption of the genes encoding CNP or its receptor, GC-B, demonstrated that this "natriuretic peptide" is unlikely to regulate renal sodium excretion under physiological conditions. Instead, it exerts important autocrine/paracrine GC-B-mediated modulatory effects on cellular proliferation and differentiation within the heart, during angiogenesis and fundamentally during bone formation. In addition, GC-B deficient mice revealed a complex phenotype, with developmental lack of bifurcation and misguidance of sensory axons within the spinal cord (Schmidt et al. 2007), seizure attacks, and female infertility (Tamura et al. 2004) observations which will stimulate further work to assess the various local, physiological functions of the CNP/GC-B system in tissue remodelling, reproduction, or even brain and neural functions.

4 Intestinal GC-C Mediates the Functions of Guanylin and Uroguanylin in the Local Homeostatic Control of Ion Transport and Cell Proliferation/Differentiation

GC-C, which contains an extracellular domain with limited sequence similarity to the above two isoforms GC-A and GC-B, is densely expressed in the apical, brush border membrane of the intestinal epithelium and represents the receptor for two endogenous intestinal peptides, guanylin and uroguanylin (Currie et al. 1992; Hamra et al. 1993). GC-C mediated increases in epithelial cGMP activate cGMP-dependent protein kinase II, which then stimulates cystic fibrosis transmembrane conductance regulator (CFTR)-dependent chloride and bicarbonate secretion, ultimately driving the paracellular movement of Na^+ into the intestinal lumen (Forte and Currie 1995). Notably, this modulatory endogenous pathway is disproportionally stimulated by exogenous heat-stable enterotoxins (STa) produced by several bacteria, which strongly activate GC-C and thereby cause secretory diarrhea (Schulz et al.

1990; Forte and Currie 1995). In fact, resistance to these pathogens is a major feature of the phenotype of GC-C-deficient mice (Table 2) (Schulz et al. 1997). In addition to these secretory effects of GC-C, different observations in vitro/in vivo demonstrated that this receptor not only regulates intestinal ion and water transport but also coordinates component processes maintaining homeostasis of the cell proliferation/differentiation within the crypt-villus axis. First several studies concentrated on the relationship between the GC-C receptor and colon cancer. GC-C signaling through CNG channel disrupts tumor cell cycle progression and proliferation (Pitari et al. 2001). Further, oral treatment with synthetic uroguanylin suppressed intestinal tumor formation in mouse models (Shailubhai et al. 2000). Strong support for a natural role for GC-C in the regulation of intestinal cell proliferation came from guanylin- and GC-C-deficient mice, both showed an expansion of the proliferating crypt compartment of the colon, i.e. increased crypt length, with increases in rapidly cycling progenitor cells and reductions in differentiated cells (Steinbrecher 2002; Li et al. 2007). In the context of the near-uniform loss of guanylin and uroguanylin expression (and thereby in GC-C/cGMP signaling) early in intestinal tumorigenesis (Steinbrecher et al. 2000), dysregulation of those homeostatic processes may contribute to mechanisms underlying colon cancer.

Besides these local functions, the (uro)guanylin/GC-C system might form an endocrine enteric-renal axis to coordinate salt ingestion with natriuresis. Hence, both peptides circulate in the bloodstream and very high concentrations of uroguanylin are excreted in urine (Hamra et al. 1993; Kuhn et al. 1993; Hess et al. 1995). Exogenous synthetic guanylin and especially uroguanylin initiates a diuretic, natriuretic, and kaliuretic response both in the isolated perfused kidney model and in laboratory animals (Santos-Neto et al. 1999; Carrithers et al. 2004). Physiological studies in genetically modified mice support this hypothesis. At difference to mice lacking guanylin (Steinbrecher et al. 2002), mice without uroguanylin apparently do not have abnormalities in intestinal epithelial proliferation. Instead, they exhibit a blunted natriuresis following enteral, but not intravenous NaCl loading (Lorenz et al. 2003). Moreover, regardless of the level of dietary salt intake, their mean arterial blood pressure under resting conditions is increased by $\sim$10–15 mmHg (Lorenz et al. 2003). Together, these findings establish a role for uroguanylin in an enteric-renal communication axis which is involved in the maintenance of salt and water homeostasis (see a comprehensive editorial by Forte 2005). However, although GC-C-mRNA is expressed in renal tubuli, the role of GC-C in this axis is very much unclear. Intriguingly, some of the renal effects of uroguanylin and guanylin persist in the GC-C-deficient mouse, indicating the existence of an as yet unidentified guanylyl cyclase or noncyclase receptor for these peptides (Carrithers et al. 2004; Sindic et al. 2005). This hypothesis is further substantiated by the observation that GC-C – deficient mice, at difference to the uroguanylin knockouts, do not exhibit increased blood pressure (own unpublished observations) or impaired renal excretion after enteral salt loading (Elitsur et al. 2006).

5 The Retinal GCs, GC-E and GC-F, Have an Important Role in Phototransduction: Mutations in the Human GC-E (RETGC-1) Gene Cause Congenital Blindness

Cyclic GMP plays a central role in the responses of rod and cone photoreceptors to light. In light, photoactivated rhodopsin stimulates PDE6 activity in rods and cones. This leads to the hydrolysis of cGMP, causing the closure of cGMP-gated (CNG) cation channels in the plasma membrane of photoreceptor outer segments. Channel closure causes the concentration of intracellular free Ca^{2+} to decline and by doing so, it stimulates resynthesis of cGMP by two membrane GCs, GC-E and GC-F (also referred to as RetGC-1 and RetGC-2) (Table 1) (Yang et al. 1995; Lowe et al. 1995). The primary structures of these retinal GCs are generally similar to those of the other particulate GCs but there are also several notable differences. Studies to detect a soluble, extracellular ligand that regulates GC-E/F activity via the ECD have been all negative. This suggests that the ECD of retinal GCs may not regulate GC activity, but it is possible that the ECD has a regulatory function that has not been detected. Instead, cGMP production by GC-E and GC-F is regulated intracellularly by two Ca^{2+}-binding GC activator proteins, GCAP-1 and GCAP-2 (Laura and Hurley 1998; Palczewski et al. 2004). GCAPs have three high affinity Ca^{2+} binding sites (EF hands). Biochemical studies suggest that GCAPs bind constitutively to the intracellular KHD of GC-E and GC-F. In the absence of Ca^{2+}, GCAPs stimulate and in the presence of Ca^{2+}, they inhibit GC activity. This allows cGMP synthesis to be determined by intracellular free Ca^{2+} levels using GCAPs as Ca^{2+} sensors (reviewed by Dizhoor and Hurley 1999). Thus, retinal GCs are not only important for maintaining the basal cGMP level in darkness but also critical for restoration of cGMP levels and reopening of CNG channels, expediting the recovery of photoreceptors following light stimulation. Interestingly, GC-F expression is confined to the retina, whereas GC-E is additionally expressed in the pineal gland and olfactory bulb (Duda and Koch 2002) as well as the choclear nerve and organ of Corti (Seebacher et al. 1999).

Genetic mouse models with ablated GC-E and/or GC-F revealed that these are the only cyclases involved in phototransduction (see Table 2) (Yang et al. 1999; Baehr et al. 2007). Selective GC-E deletion led to cone-specific dystrophy and paradoxical responses of rods to light (Yang et al. 1999). Selective GC-F deletion did not provoke significant alterations in electroretinographic responses (Baehr et al. 2007). Deletion of both GCs results in an animal model without rod and cone vision and a phenotype of recessive cone/rod dystrophy, demonstrating that retinal GCs are necessary not only for normal photoresponses but also for photoreceptor viability (Baehr et al. 2007). Biochemical studies in these mice suggest that retinal GCs may be involved in the trafficking of proteins of the phototransduction cascade and also may have a structural role in stabilizing the rod and cone disk membranes (Karan et al. 2008).

In the human retina, the two isoforms are encoded by the *RETGC-1* and *RETGC-2* genes, with *RETGC-1* showing higher levels of expression in cone

than in rod cells. Recessive mutations in *RETGC-1*, found throughout the catalytic and kinase-like domains, cause Leber's congenital amaurosis type 1 (LCA1), an early onset rod/cone dystrophy that causes blindness from birth (Perrault et al. 1996; Kelsell et al. 1998). Thus, it is unlikely that *RETGC-2* can compensate for the loss of *RETGC-1* activity in human retina. Interestingly, a latter study identified two new mutations in the *RETGC-1* gene that are associated with dominant cone-rod dystrophy, resulting in early loss of central vision, and peripheral field loss by the fourth decade of age (Smith et al. 2007). Both mutations result in single amino acid substitutions within the hinge region, the putative dimerization domain of the *RETGC-1* protein. This may lead to a steric change during dimer formation that affects the GC activity of both mutant-mutant and mutant-normal dimers. No human retinal disease has yet been linked to a defect in the GC-F (*RETGC-2*) gene. The *RETGC-1* mutations were the first examples of cone-rod dystrophy arising from structural changes in one of the enzymic components of the phototransduction process.

6 Contribution of GC-D to Chemosensory Function in the Olfactory Epithelium of Rodents

The last two membrane GCs to be referred, GC-D and GC-G, appear to be pseudogenes in the human (Potter 2005). Intriguingly, the expression of GC-D in rodents is restricted to a small population of neurons within a single topographic zone in the olfactory neuroepithelium (Fülle et al. 1995). Besides GC-D, these neurons express specific cGMP-regulated third messengers, namely PDE2 (Julifs et al. 1997) and a CNG channel subunit, CNGA3 (Leinders-Zufall et al. 2007), all three proteins being colocalized in olfactory cilia, where odorant signaling is initiated. Instead, they lack key components of the canonical odor transduction cascade, involving cAMP and calcium. In contrast to most other olfactory neurons, these neurons appear to project to a distinct group of 12–13 specialized glomeruli in the olfactory bulb that are similar to the subset that have been termed "necklace glomeruli" (Julifs et al. 1997). The function of these glomeruli is not known, but the mediation of some responses to suckling pheromones has been suggested. Mice with genetic deletion of GC-D were recently generated (Table 2; Leinders-Zufall et al. 2007) and did not exhibit obvious defects in suckling or mating effectiveness. Instead, by combining patch clamp recording and confocal Ca^{2+} imaging from single dendritic knobs in situ, the authors found that GC-D cells recognize the peptide hormones, guanylin and uroguanylin as well as natural urine stimuli. This was surprising because guanylins were previously considered specific and exclusive ligands of GC-C (see Sect. 5). Leinders-Zufall and colleagues (2007) convincingly demonstrated that synthetic guanylin and uroguanylin stimulate an excitatory, cGMP-dependent signaling cascade that increases intracellular Ca^{2+} and action potential firing in the olfactory epithelium, these responses being abolished both in GC-D- and in CNGA3-deficient mice. Because these "intestinal natriuretic peptides" have been implicated in the modulation of intestinal and renal salt and water transport (see Sect. 5), and

because they are excreted in feces (guanylin) and urine (especially uroguanylin), the authors hypothesize an association with the detection of information related to the metabolic status, specifically to regulatory mechanisms that mediate the integration of salt and water balance in areas of the central nervous system such as the hypothalamus. A very recent study confirmed the activation of GC-D by uroguanylin, questionated its activation by guanylin, and proposed a second mechanism of intracellular (not extracellular) activation of GC-D by Ca^{2+}-neurocalcin (Duda and Sharma 2008). The exact physiological function of GC-D ultimately remains to be determined.

7 GC-G: An Orphan Receptor with a Deleterious Role in Renal Pathophysiology

GC-G is the last member of the family of mammalian membrane GCs. It was described by David L. Garbers and colleagues 10 years ago (Schulz et al. 1998). No other mammalian transmembrane GCs are predicted on the basis of gene sequence repositories. Ligands for the other GC receptors failed to stimulate GC-G expressed in transient or stable cells. Based on Northern hybridization, GC-G has a broad tissue distribution in the rat, including the lung, intestine, and skeletal muscle (Schulz et al. 1998). In contrast, in mice GC-G is highly enriched in sperm (Huang et al. 2006), where it apparently modulates the Ca^{2+} influx/efflux and thereby motility responses, and in the kidney (Lin et al. 2008), where the physiological role is largely unknown. Mice with genetic disruption of GC-G do not exhibit functional defects under "resting" conditions (Table 2). However, they are protected against ischemic renal damage and failure. Because ischemia-reperfusion markedly induced the tubular epithelial expression of GC-G in kidneys of wild-type mice, the authors suggested that the induction/activation of GC-G may play a role under pathophysiological conditions, contributing to tubular damage and renal failure through apoptosis and inflammation (Lin et al. 2008). Hence, the physiological regulation and function of GC-G is largely unknown.

8 Conclusions and Future Directions

Although the observations in rodent systems cannot be directly extrapolated to human beings, the application of gene targeting technology in mice has provided valuable information regarding the molecular physiology and diverse biological functions of membrane GC receptors and their ligands. They also pointed out the potential implications of specific dysfunctions in these systems for human diseases. In fact, as reviewed in this paper, in two cases specific links between mutations in membrane GCs (GC-B, GC-E) and human congenital diseases (dwarfism, blindness) was latter found and reemphasized the value of genetic mouse models.

This paper will conclude with some of the important, as yet unanswered, questions that relate to membrane GCs and their cognate ligands. The processes regulating GC receptor responsiveness in vivo, especially those mediating phosphorylation (sensitization) and dephosphorylation (desensitization) of GC-A, GC-B, and possibly of other receptor GCs are largely unknown. The discrepant phenotypes observed in some ligand- and respective receptor-knockout mouse models (BNP vs. GC-A, uroguanylin vs. GC-C) suggest the existence of as yet unidentified receptors. The in vivo relevance of recent in vitro observations showing a ligand-independent direct activation of receptor GCs by intracellular molecules (of GC-A, D, E, F by PAK1; of GC-D by neurocalcin) is unknown. What are the mechanisms by which GC-A protects the heart from hypertrophy and fibrosis and how does GC-B regulate skeletal growth? Is GC-C a therapeutical target to significantly change the progression of certain intestinal cancers? The physiological functions of GC-D and GC-G are largely unknown. And finally, is cGMP the only means by which the cyclases signal? Clearly, this is an exciting field of (patho)physiological and clinical importance with many answers but even more questions.

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Phosphodiesterases in the Central Nervous System

Thomas Kleppisch

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Abstract Phosphodiesterases (PDEs) represent important cornerstones of cGMP signaling in various tissues. Since the discovery of PDE activity in 1962, it has become clear that the functional characteristics of PDEs and their role in cyclic nucleotide signaling are fairly complex. On the one hand, members of the PDE family responsible for the hydrolysis of cGMP affect cellular responses by shaping cGMP signals derived from the activation of soluble cytosolic and/or membrane bound particulate guanylyl cyclases. Conversely, PDEs may function as downstream effectors in the cGMP signaling cascade. To make things even more sophisticated, cGMP modulates the activity of several PDEs either directly, by binding to a regulatory domain, or indirectly, through phosphorylation, and the result can be either inhibition or stimulation of the enzyme, depending on the subtype. Furthermore, cross-talk between cGMP and cAMP signaling is achieved by cGMP-dependent modulation of PDEs hydrolyzing cAMP and vice versa. Mammals posses at least 21 PDE genes

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and often express a set of PDEs in a tissue- and differentiation-dependent manner. Given these premises, it is still a challenging task to elucidate the physiological function(s) of individual PDE genes. The present chapter focuses on the role of PDEs as regulators of neuronal functions. Useful information regarding this topic has been gained by studying (1) the expression pattern of PDEs in the CNS, (2) the association of PDEs with specific macromolecular signaling complexes and (3) the phenotypes associated with mutations or ablation of PDE genes in man, mice and fruit flies, respectively. PDEs degrading cGMP and/or being regulated by cGMP have been implicated in cognition and learning, Parkinson's disease, attention deficit hyperactivity disorder, psychosis and depression. Correspondingly, modulators of PDEs have become attractive tools for treatment of these disorders of CNS function.

Keywords: Cyclic nucleotides · Phosphodiesterases · CNS (cental nervours system) · Synaptic plasticity · LTP (long-term potentiation) · LTD (long-term depression) · Learning · Memory · Behaviour

1 Introduction

The concept of cyclic nucleotides, cAMP and cGMP serving as intracellular second messengers in the signal transduction of chemical or physical extracellular cues into cellular responses, which was initially met with skepticism, has now become the basis for understanding a large variety of physiological functions. Indeed, cGMP is involved in the regulation of processes as diverse as the contraction of cardiac and smooth muscle, platelet aggregation, secretion, immune cell response and inflammation, neuronal excitability and synaptic plasticity (for review see Feil et al. 2005; Hofmann et al. 2006; Conti and Beavo 2007; Feil and Kleppisch 2008). Soluble guanylyl cyclase (sGC) and a class of membrane bound particulate guanylyl cyclases (pGC) are the principal sources of cGMP (see chapters by Kuhn et al., Marletta et al. and Koesling et al., this volume). Depending on cell type and function, changes in cytosolic concentration of cGMP last for a very short time (e.g. milliseconds in the outer segment of retinal photoreceptors) or up to several hours (e.g. in endocrine cells under the influence of trophic hormones). The shape of the cGMP transient crucially depends on the function of cyclic nucleotide phosphodiesterases (PDEs), the activity of which was described soon after the discovery of cyclic nucleotides in the late 50s and early 60s of the twentieth century (Rall and Sutherland 1958; Ashman et al. 1963). Since then, an amazing complexity of the PDE system has emerged, both on the structural and functional levels. The current list of mammalian PDEs comprises at least 11 gene families with 21 genes. Their activities are subject to regulation by diverse biochemical mechanisms including phosphorylation/dephosphorylation, allosteric binding of cGMP or cAMP, binding of Ca^{2+}/calmodulin and various protein–protein interactions (Conti and Beavo 2007; see also chapter by S. Francis et al., this volume). As a result of these multifaceted levels of PDE regulation, cyclic nucleotide levels are kept within

narrow ranges of concentration. Modern concepts suggest microdomains of cyclic nucleotide signaling, i.e. three-dimensional modulation of the cGMP signal may be achieved by organizing PDEs into macromolecular complexes with (1) regulatory proteins (e.g. kinases), (2) anchoring or scaffold proteins and (3) other signaling proteins, and is thought to support the specificity of cyclic nucleotide effects in a particular tissue (for review see Bender and Beavo 2006). Most cell types express a set of PDEs that alters during ontogenesis and with differentiation (Conti and Beavo 2007). Nonetheless, there is evidence linking the dysfunction of individual PDE genes to diseases in the endocrine, cardiovascular and nervous systems. This chapter reviews the expression of PDEs and their functions and possible clinical implications, with regard to the CNS. Owing to the topic of the present volume, emphasis is put on PDEs hydrolyzing cGMP and/or being regulated by cGMP.

2 Classification, Substrate Specificity and Modulation of Phosphodiesterases

This volume already includes a comprehensive description of the nomenclature biochemistry and physiology of cyclic nucleotide phosphodiesterases (PDEs) by S. Francis and coauthors. Therefore, this section will review the corresponding literature very briefly.

Class I PDEs found in mammals are a superfamily of phosphohydrolases selectively catalyzing the hydrolysis of the 3′ cyclic phosphate bonds in adenosine and/or guanosine 3′, 5′ cyclic monophosphates (Fig. 1a). The human genome comprises 21 genes encoding PDEs that have been grouped into 11 gene families (PDE1–11) based on their sequence homologies and functional characteristics (for review see Bender and Beavo 2006; Conti and Beavo 2007). A vast number of PDE transcripts arise from alternative splicing and the use of alternative transcription start sites. The latter results from the complex structure of most PDE genes with multiple promoters. Major structural, biochemical, pharmacological and functional characteristics of these gene families are summarized in Table 1. By their substrate specificity, all PDE families fall into one of the following three categories: (1) cAMP specific, (2) cGMP specific or (3) PDEs with dual enzymatic activity (Fig. 1b). In addition to their primary effect on the level of cyclic nucleotides, PDEs enable cross-talk between cGMP and cAMP signaling, e.g. through cGMP-dependent modulation of the activity of PDEs catalyzing the hydrolysis of cAMP (Fig. 1c). Similarly, cAMP may slow down the degradation of cGMP by PDE10. In case of the PDE5, cGMP stimulates phosphorylation of the enzyme by cGK, which results in enhanced hydrolysis of cGMP and facilitates termination of the cGMP signal (Rybalkin et al. 2002; Shimizu-Albergine et al. 2003; Koesling et al. 2005). This cGMP-mediated negative feedback is also thought to be responsible for desensitization of PDE5 (Koesling et al. 2005). Ultimately, it has to be emphasized that concentrations of cyclic nucleotides in a microdomain are dynamic functions of the activities of various cyclases and PDEs within a particular cellular compartment.

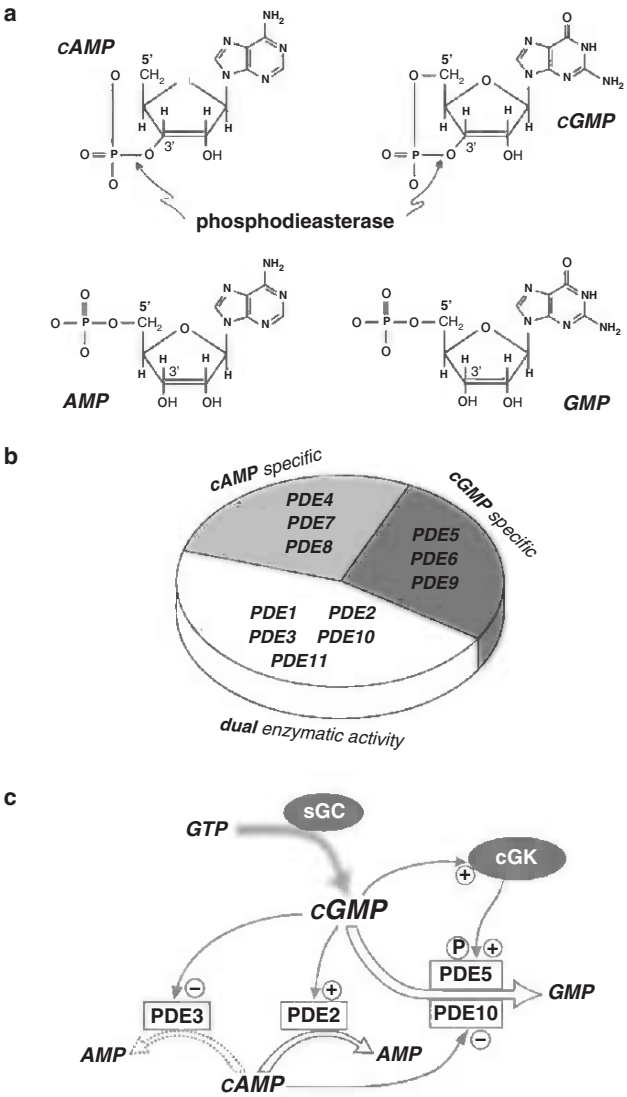

Fig. 1 Role of phosphodiesterases (PDEs) in cyclic nucleotide signaling. a Phosphodiesterases (PDEs) of the mammalian superfamily hydrolyze the 3′ cyclic phosphate bond of the intracellular second messenger(s) cAMP and/or cGMP yielding inactive 5′-AMP and/or 5′-GMP. **b** Breakdown of cAMP and cGMP can be catalyzed by PDEs highly specific either for cGMP (PDE5, PDE6, PDE9) or cAMP (PDE4, PDE7, PDE8). Some PDEs possess dual enzymatic activity and catalyze the hydrolysis of both cAMP and cGMP (PDE1, PDE2, PDE3, PDE10, PDE11). **c** Roles of PDEs in cGMP signaling. Some PDEs hydrolyze cGMP, e.g. the cGMP-specific PDE5. cGMP accelerates its PDE5-dependent degradation by stimulating cGK and phosphorylation of the PDE5. Cross-talk between cGMP and cAMP signaling is achieved e.g. through cGMP-mediated stimulation and inhibition of the cAMP-hydrolyzing PDE2 and PDE3, respectively. cAMP can bind to a GAF domain (Gross-Langenhoff et al. 2006) of PDE10 and inhibit hydrolysis of cGMP in vitro (Conti and Beavo 2007). **sGC**: soluble guanylyl cyclase; **cGK**: cGMP-dependent protein kinase

Table 1 Structural, biochemical, pharmacological and functional properties of mammalian phosphodiesterases

Subfamily Isoform(s)	Substrate specificity	Regulatory mechanism	GAF domain	Expression in the CNS
PDE1 *A, B, C*	cGMP/cAMP	Ca^{2+}/calmodulin/ stimulation		Cortex, hippocampus, striatum, olfactory epithelium (PDE1C2)
PDE2 *A*	cAMP/cGMP	cGMP/stimulation	+	Cortex, hippocampus, striatum
PDE3 *A, B*	cAMP/cGMP	cGMP/inhibition phosphorylation by PKB/Akt and PKA stimulates activity		Hippocampus, striatum
PDE4 *A, B, C, D*	cAMP	phosphorylation by ERK		Neocortex, hippocampus, striatum, olfactory system, area postrema
PDE5 *A*	cGMP	cGMP/stimulation by cGK-mediated phosphorylation	+	Spinal cord, cerebellar Purkinje cells
PDE6 *A, B, C*	cGMP		+	Retinal photoreceptors, pineal gland
PDE7 *A, B*	cAMP			Hippocampus, striatum, cerebellar Purkinje cells
PDE8 *A, B*	cAMP			Cortex, striatum, hippocampus
PDE9 *A*	cGMP			Cortex, hippocampus, olfactory system, basal ganglia, thalamus, cerebellar Purkinje cells
PDE10 *A*	cGMP/cAMP	cAMP/inhibition phosphorylation by PKA alters subcellular distribution	+	Striatum, pituitary gland
PDE11 *A*	cAMP/cGMP		+	

GAF: cGMP-activated PDEs, Adenylyl cyclase, and Fh1A; PKA: cAMP-dependent protein kinase A; cGK: cGMP-dependent protein kinase

3 Expression of Phosphodiesterases in the CNS

The following considerations recall the difficulties in getting a comprehensive picture of the expression of PDEs: (1) as discussed above, there is a vast diversity of PDE transcripts; (2) the expression levels (i.e. protein concentrations) required for a functional homeostasis of cyclic nucleotide concentrations are usually low because of the high catalytic activity of many PDEs; (3) expression of PDEs in a particular cell type may alter depending on various factors including developmental and proliferational status, grade of excitation, drug treatment and disease (Naro et al. 1996;

Rybalkin et al. 1997; Takahashi et al. 1999, 2001; Jin and Conti 2002; Perez-Torres et al. 2003; Hebb et al. 2004; Reyes-Irisarri et al. 2007b, 2008; Dlaboga et al. 2008). Nevertheless, it is now clear that literally all cells express PDEs, usually a set from different gene families or variants from the same family. Moreover, virtually all the PDEs are expressed somewhere in the CNS and, as a result of appealing functional findings, have become promising targets for drug development (for review see Menniti et al. 2006). The following paragraphs shortly review neuronal expression of the three major subdivisions of PDEs based on their substrate specificity (cf. Fig. 1b).

3.1 cGMP-Specific PDEs

PDE5 was first isolated from platelets and lung characterized by high expression and is localized in the cytosol. Studies with neuronal tissue showed that the PDE5 is expressed in the spinal cord, pyramidal cells of the hippocampus and most abundantly in the cerebellum, specifically the Purkinje cells, while other parts of the brain show negligible or only marginal expression of the enzyme (Kotera et al. 1997, 2000; Loughney et al. 1998; Giordano et al. 2001; Shimizu-Albergine et al. 2003; Bender and Beavo 2004; van Staveren et al. 2004; de Vente et al. 2006; Reyes-Irisarri et al. 2007a).

Similar to the pattern of PDE5, highly abundant expression of the cGMP-hydrolyzing PDE6 is found in a specific type of neuron. PDE6 is best known for its function in retinal photoreceptors, where PDE6A/PDE6B is found in rods and PDE6C in cones (cf. Bender and Beavo 2006). PDE6 concentration in these cells can reach 10–50 μM and thus exceed the free concentration of cGMP by one order of magnitude (Cote 2006). In addition, PDE6A/B has been detected in the pineal gland. Notably, PDE6 can be targeted to the membrane by isoprenylation, and its expression in non-retinal tissue may be functionally relevant for/during embryonic development.

Differing from the picture for PDE5 and PDE6, PDE9 appears to be expressed widely throughout the brain, e.g. in neurons of the dentate gyrus and hippocampal pyramidal cells, in the olfactory system, in the neocortex, basal ganglia, thalamus and the cerebellar Purkinje cell layer (Andreeva et al. 2001; Van Staveren et al. 2003, 2004; Reyes-Irisarri et al. 2007a). PDE9 expression is believed to be predominantly neuronal, despite some expression in astrocytes (van Staveren et al. 2002). The PDE9 isoforms are commonly cytosolic proteins, except for PDE9A1, which is specifically localized to the nucleus (Bender and Beavo 2006).

3.2 cAMP-Specific PDEs

The cAMP-specific PDE4s represent the mammalian homologs of the gene product defective in the *Drosophila melanogaster* mutant *dunce*, which exhibits a marked learning impairment. PDE4 has four splice variants (A–D) that are differentially

distributed throughout the human brain. PDE4A, PDE4B and PDE4D are widely distributed in the human brain and have been detected in the olfactory system, the neocortex, the hippocampus, the amygdala, the striatum, the granular layer of the cerebellum and the area postrema (Engels et al. 1995; Perez-Torres et al. 2000; Menniti et al. 2006; Reyes-Irisarri et al. 2008). In contrast, PDE4C appears to be restricted to the cortex, cerebellum and thalamus. The subcellular distribution of PDE4s varies greatly depending on interaction(s) with other proteins targeting them to macromolecular signaling complexes (Houslay and Adams 2003).

Isoforms of the cAMP-hydrolyzing PDE7 are expressed in neurons of the olfactory tubercle, caudate-putamen, thalamus, neocortex, hippocampus, cerebellum and the area postrema with PDE7B as the major form (Reyes-Irisarri et al. 2005; Bender and Beavo 2006; Menniti et al. 2006). Both PDE7A and PDE7B are found in the cytosolic fraction.

cAMP-specific PDE8, specifically PDE8B, is uniquely and abundantly expressed in many brain regions (except the cerebellum) e.g., in the neocortex, striatum and hippocampus (Kobayashi et al. 2003; Bender and Beavo 2006; Menniti et al. 2006). PDE8s can be localized in the cytosol or membrane-bound.

3.3 PDEs with Dual Enzymatic Specificity

Individual PDE1 isoforms are selectively expressed in specific tissues and cell types due to a tight regulation by numerous factors which are exemplified by the nervous system: all three PDE1 isoforms are expressed in the brain and peripheral neurons but to greatly differing degrees, depending on the region. For example, PDE1C2 is highly localized to the olfactory epithelium where it is thought to play an important role in rapid regulation of cAMP responses to odorants (Yan et al. 1994, 1995; Menniti et al. 2006). PDE1A and PDE1B are expressed in neurons of the neocortex, hippocampal pyramidal cells and striatal neurons (Menniti et al. 2006). Interestingly, PDE1 expression differs, depending not only on the region, but also within a neuronal population of the same region: e.g. PDE1B is highly expressed in some but not all cerebellar Purkinje neurons (Shimizu-Albergine et al. 2003; Bender and Beavo 2004). The majority of PDE1 isoforms appear to be localized to the cytosol.

Of the three known PDE2s, PDE2A1 is cytosolic, whereas PDE2A2 and PDE2A3 are membrane-bound. Expression of these PDE2s appears strongly localized to unique neuronal populations and brain regions, including the hippocampus, neocortex and striatum (Bender and Beavo 2004, 2006; Menniti et al. 2006).

The two PDE3 variants, PDE3A and PDE3B, contain a large and a small hydrophobic domain at their N-termini and can be either membrane-associated or cytosolic (Bender and Beavo 2006). In native tissues, PDE3B is commonly found particulate, whereas PDE3A can be both cytosolic and particulate. Expression of these PDE3s has been reported for neurons in the striatum and hippocampus (Menniti et al. 2006).

Several detailed studies of PDE10A localization in the brain (Fujishige et al. 1999; Loughney et al. 1999; Soderling et al. 1999; Seeger et al. 2003) document relatively high levels of expression. The level of mRNA transcript is particularly high in striatal neurons. However, transcripts were also found in the cerebellum, thalamus, hippocampus, spinal cord and the pituitary gland. The PDE10A2 variant is found largely in the particulate fraction of the cell.

Generally, PDE11 has not been detected in the brain. Loughney et al. (2005) have reported expression of the PDE11A4 variant in the pituitary.

4 Physiology and Pathophysiology of Phosphodiesterases in the CNS

The list of functions associated with PDEs in the nervous system is increasing. For quite a while, it has been known that PDEs play an important role in the physiology of phototransduction, olfactory and gustatory perception, other functions have become apparent only recently, with findings linking dysfunction of PDE genes to human diseases or due to phenotypes observed in animal models lacking an individual PDE. The physiology of phototransduction and olfaction is the subject of another chapter in this volume (see chapter by Biel and Michalakis). Therefore, the focus here is specifically on findings regarding the functional role of PDEs in learning and synaptic plasticity and the pathophysiology of various psychiatric and neurological disorders. These findings lay down a basis for potential new approaches in the treatment of the respective disease and have, accordingly, stimulated major efforts in the search for selective modulators of individual PDEs involved.

4.1 PDEs in Learning and Synaptic Plasticity

Drugs currently used for the treatment of age-related cognitive impairments have only limited efficacy and there is a need for alternative therapeutic approaches, e.g., targeting second messenger systems utilized by neurotransmitters and involved in learning and memory. The two second messengers cGMP and cAMP play a major role in biochemical cascades believed to regulate these cognitive processes, and growing evidence indicates a modulatory function of PDEs in this context (see Table 2). Initial findings date back to 1976 when a Drosophila mutant, *dunce*, was first described. Drosophila normally learn to avoid an odorant associated with an electric shock, but this learning is impaired in fruit flies with a defective *dunce* gene (Dudai et al. 1976). This gene highly expressed in mushroom bodies was later identified as a cAMP phosphodiesterase homologous to mammalian PDE4s (Byers et al. 1981; Davis and Kiger 1981; Kauvar 1982; Chen et al. 1986; Qiu et al. 1991; Qiu and Davis 1993; Engels et al. 1995). Work in mammals also supports the view that members of the PDE4 family, by regulating specific cAMP pools, control neuronal signaling cascades important for learning and memory (Ahmed and Frey 2005; Rose

Table 2 CNS phenotypes related to ablation and mutations of PDE genes or genes controlling the function of PDEs

Species	Model	Mutant gene/ gene locus	Associated phenotype or disease	References
Mouse	Knockout	PDE1B	Increased locomotor activity, increased dopamin receptor-mediated phosphorylation of DARP-32, spatial learning deficit	(Reed et al. 2002)
Drosophila melanogaster	Spontaneous mutation	*dunce* (homologue of PDE4)	Learning deficit	(Dudai et al. 1976; Byers et al. 1981; Tully 1991)
Mouse	Knockout	PDE4B	Defect in prepulse inhibition, decreased baseline motor activity, exaggerated locomotor response to amphetamine	(Jin et al. 2005; Siuciak et al. 2008)
Human	Spontaneous mutation	PDE4B and DISC1	Schizophrenia susceptibility	(Millar et al. 2005; Thomson et al. 2005)
Mouse	Knockout	PDE4D	Not examined due to neonatal lethality	(Jin et al. 1999)
Human	SNP in non-coding region		Stroke susceptibility	(Gretarsdottir et al. 2002; Gretarsdottir et al. 2003)
Mouse	*rd* spontaneous mutation	PDE6B	Retinal degeneration	(Fletcher et al. 1986; Pittler and Baehr 1991; Cote 2006)
Human	Spontaneous mutation	PDE6B	Retinal degeneration, autosomal recessive retinitis pigmentosa, congenital stationary night blindness	(for review see Farber and Danciger 1997)
Human	SNPs	PDE9A	Major depression and bipolar affective disorder	(Straub et al. 1994; Menniti et al. 2006 and refs. therein; Wong et al. 2006)
Mouse	Knockout	PDE10A	Increased escape latency in the Morris water maze[a], impaired conditioned avoidance learning, reduced spontaneous locomotor activity, increased social interaction, increased levels of striatal cAMP	(Siuciak et al. 2006b; Sano et al. 2008)
Human	SNPs	PDE11A	Major depression	(Wong et al. 2006)

[a] This finding may suggest a spatial learning deficit, but may also be due to reduced swimming activity of PDE10A null mutants.

et al. 2005; Blokland et al. 2006; Menniti et al. 2006). In rats for example, rolipram, a prototypical PDE4 inhibitor, attenuated scopolamine-induced deficits in spatial learning (8-arm radial maze) and passive avoidance response (Egawa et al. 1997), and improved the performance in the object recognition task (Rutten et al. 2007). Moreover, rolipram improves contextual fear learning in wild type mice (Barad et al. 1998) and ameliorates deficits of a mouse model of Alzheimer's disease in this hippocampus-dependent learning task (Gong et al. 2004). A study in macaques demonstrates that rolipram can improve object retrieval performance (Rutten et al. 2008). On the other hand, impairment of learning in a water maze task by rolipram has been reported to be caused by up-regulation of the PDE4D gene and a subsequent persistent decrease of cAMP concentration (Giorgi et al. 2004). The time dependency of the rolipram effects on behavior suggested that PDE4s are involved in memory acquisition and consolidation, i.e. the protein synthesis-dependent conversion of short-term into stable long-term memory. According to a generally accepted view, cAMP promotes the latter process by activating the transcription regulator cAMP response element-binding protein (CREB) (for review see Kandel 2001; Lonze and Ginty 2002). Remarkably, rolipram can rescue mice carrying a mutation leading to decreased activation of the CREB/CRE signaling cascade from a defect in long-term memory (Bourtchouladze et al. 2003). Long-term potentiation (LTP) and long-term-depression (LTD) are two forms of synaptic plasticity believed to share important properties with cellular events underlying learning and memory (for details see Malenka and Bear 2004 and the chapter of Kleppisch and Feil, this volume). Intriguingly, long-term potentiation and CREB phosphorylation in the hippocampus are facilitated by rolipram (Barad et al. 1998; Navakkode et al. 2004; Monti et al. 2006). In line with the behavioral effects discussed above, rolipram diminished deficits in LTP in a mouse model of Alzheimer's disease (Gong et al. 2004). Finally, the PDE4 selective inhibitor has been reported to convert decremental early LTD into protein biosynthesis-dependent long-lasting LTD with mitogen-activated protein kinase being a key element in the signaling cascade mediating this effect (Navakkode et al. 2005).

There is also substantial evidence demonstrating a modulatory function of cGMP-specific PDEs for memory and synaptic plasticity (for review see Blokland et al. 2006; Menniti et al. 2007), which is not surprising, given that increases of intracellular cGMP facilitate these processes (see chapter of Kleppisch and Feil, this volume). Thus, selective inhibitors of PDE5 have been reported to attenuate impairment of rats in maze-learning tasks, induced by inhibition of NO synthase (Devan et al. 2006, 2007), enhance object recognition memory in rats and mice (Prickaerts et al. 2002b, 2004; Rutten et al. 2005, 2007), retention of an inhibitory avoidance response in mice (Baratti and Boccia 1999) and object retrieval performance in macaques (Rutten et al. 2008). Most of these effects are dependent on the time point of drug administration, leading to the conclusion that cGMP modulates specifically earlier phases of the memory consolidation process (cf. Prickaerts et al. 2002a). However, a study of hippocampal LTP with zaprinast, an inhibitor of PDE5 and PDE6, suggested the functional relevance of a negative feedback of cGMP (Monfort et al. 2004) involving activation of cGK and PDE, likely PDE5. A role

of PDE5 in hippocampal LTP has also been supported by the following findings of Kuenzi and coworkers (2003): mice with a loss of function mutation in the PDE6B gene show normal LTP, and zaprinast induces a sustained depression of synaptic transmission to the same extent in both wild-type and mutant mice. PDE5 has also been implied in cerebellar LTD (Shimizu-Albergine et al. 2003), while LTD in corticostriatal synapses can be induced chemically with zaprinast (Calabresi et al. 1999).

PDEs with dual enzymatic activity have also been shown to regulate memory function. Mice with a targeted ablation of the PDE1B display a spatial learning deficit in the Morris water maze, which may be related to altered dopaminergic signaling via the cGK substrate DARP-32 (Reed et al. 2002). The PDE2 inhibitor BAY 60-7550 has been reported to improve memory in the social and object recognition task (Boess et al. 2004; Rutten et al. 2007). Mice with a genetic ablation of the striatum-enriched PDE10A required additional training sessions to reach the same level of proficiency as their wild-type litter mates in a conditioned avoidance task (Siuciak et al. 2006b). Mice lacking PDE10A also showed increased escape latency in the Morris water maze (MWW) test. However, it remains unclear whether this finding reflects a spatial learning deficit and not merely a reduced swimming activity of the mutants. Supporting the latter view, chronic treatment of wild-type mice with papaverine, a selective PDE10A inhibitor, causes no marked disturbances in the MWW (Hebb et al. 2008). A possible function of PDE10 in synaptic plasticity was suggested based on data from in situ hybridization analysis, showing that expression of a specific subset of PDE10A splice variants increases following LTP (O'Connor et al. 2004). Owing to the dual enzymatic activity of PDE1B, PDE2 and PDE10, it is difficult to deduce which of the findings discussed are mediated through cAMP and cGMP signaling cascades, respectively. Data from Cos7 cells transfected with PDE10A3 suggest an effect on activity-dependent elevation of cGMP (O'Connor et al. 2004), while cAMP was increased in the striatum of PDE10A2 null mutants (Sano et al. 2008).

4.2 PDEs in Affective Disorders and Schizophrenia

Pharmacological and genetic evidence suggest a role of various PDEs in the pathogenesis of psychiatric illness such as affective disorders and schizophrenia (Berton and Nestler 2006; Menniti et al. 2006; Hebb and Robertson 2007). For instance, the selective PDE4 inhibitor rolipram has been reported to exert antidepressant effects in humans and animal models of depression (Zeller et al. 1984; Zhang et al. 2002; D'Sa et al. 2005). cAMP-dependent induction of BDNF synthesis and hippocampal neurogenesis via CREB is believed to be of major importance for these effects (Nakagawa et al. 2002; Duman 2004). Antidepressant effects have also been attributed to down-regulation of PDE4B in a model of chronic nicotine treatment (Polesskaya et al. 2007). On the other hand, therapeutic effects of various antidepressant agents appear to involve regulatory changes of PDE4 activity. Thus, chronic

administration of desipramine and fluoxetine resulted in enhanced expression of PDE4A and PDE4D in cortical areas and the hippocampus (D'Sa et al. 2002, 2005; Dlaboga et al. 2006, 2008). Interestingly, similar changes have been observed with rolipram treatment and for some other PDEs. Further support for a role of PDE4 in depression provided the finding that PDE4D knockout mice display decreased duration of immobility (used as an index of behavioral despair) in the forced-swim test, i.e. loss of PDE4D activity has an antidepressant-like effect (Zhang et al. 2002). Rolipram had no antidepressant-like effect in these mice. In contrast, desipramine and fluoxetine produced similar antidepressant-like effects in wild-type and PDE4D-deficient mice, indicating the relevance of further signaling mechanisms unrelated to the activity of PDE4D. Besides antidepressant properties, anxiolytic effects of the PDE4 inhibitor rolipram in the elevated plus maze test have been described: rats show increased time spent in the open arms and number of entries to the open arms following application of the drug, despite a reduction of the overall locomotor activity (Silvestre et al. 1999). Somewhat divergent from PDE4D, PDE4B normally appears to suppress anxiety as evidenced by an anxiogenic-like behavioral phenotype in PDE4B knockout mice (Zhang et al. 2008). Despite the encouraging findings discussed, clinical applications of non-selective PDE4 inhibitors such as rolipram are limited, owing to their side effects such as nausea and emesis, which are thought to arise from effects in the gut or the area postrema. The development of subtype-selective PDE4 inhibitors may provide an approach to circumvent this problem in the future.

Direct and indirect evidence from genetic studies suggest that additional members of the PDE family are associated with an increased susceptibility for affective disorders (Straub et al. 1994; Wong et al. 2006; Hebb et al. 2008). Straub and coworkers identified a vulnerability locus for bipolar affective disorder on chromosome 21q22.3 (Straub et al. 1994). Remarkably, the gene encoding the human PDE9A enzyme has been mapped to exactly the same chromosomal locus (Guipponi et al. 1998), suggesting a link between the functional status of PDE9A and affective disorders. Similarly, a study in which SNPs in the 21 human PDE genes were genotyped suggested that polymorphisms in the genes encoding PDE9A and PDE11A increase the risk of depression (Wong et al. 2006). Future experiments with specific inhibitors of PDE9A or PDE11A, and knockout mice lacking the PDE9 or PD11A could provide the direct proof for an association of these enzymes with depression. Finally, increased anxiety has been observed in the course of pharmacological inhibition of the PDE10A isoform with papaverine (Hebb et al. 2008).

Inhibition of the striatum-enriched PDE10A may also offer a new approach for the treatment of schizophrenia (Menniti et al. 2006; Schmidt et al. 2008) with improved efficacy on negative symptoms and cognitive impairments, and fewer side effects than neuroleptics currently in use. Accordingly, treatment with the PDE10A-selective inhibitor papaverine has been reported to attenuate deficits in attention and working memory induced by subchronic systemic administration of phencyclidine, an animal model for schizophrenia (Rodefer et al. 2005). Moreover, papaverine potentiates haloperidol-induced catalepsy, having no effect when administered alone, and inhibits conditioned avoidance in rats and mice (Siuciak et al. 2006a). These

effects indeed result from selective inhibition of PDE10A as confirmed in PDE10A knockout mice.

Recent findings also link the PDE4 to schizophrenia. The strongest genetic evidence was provided by several studies in humans (Harrison and Weinberger 2005; Millar et al. 2005; Porteous et al. 2006). A group led by Porteous reported that a translocation in the gene encoding the PDE4B is selectively associated with schizophrenia in a family and results in about 50% reduction in expression of PDE4B1 protein. More intriguingly, PDE4B binds to the adapter protein DISC1 (for disrupted in schizophrenia 1), which had been previously identified by the same group as another important susceptibility marker for schizophrenia as well as affective disorders (Thomson et al. 2005; Chubb et al. 2008). Normally, interaction of PDE4B with DISC1 is terminated by an increase of cAMP via activation of PKA, which ultimately increases PDE4B activity. This mode of physiological regulation is apparently impaired by mutations in the DISC1 and PDE4B genes that disrupt interaction and cAMP-dependent regulation. Finally and in line with a possible therapeutic action in schizophrenia, the PDE4-selective inhibitor rolipram has been reported to exert neuroleptic effects comparable to those of risperidone and to reverse amphetamine-induced disruption in auditory evoked potentials in rats (Maxwell et al. 2004; Becker and Grecksch 2008).

4.3 PDEs in Neurodegenerative Disease

It is well recognized that loss of PDE activity not only affects neuronal functions, but may also have an impact on neuronal cell survival and thus represents a mechanism causing neurodegeneration (Menniti et al. 2006; Hebb and Robertson 2007). A well-known example is retinitis pigmentosa, where a single nucleotide mutation in the gene encoding the cGMP-hydrolyzing PDE6, which is highly expressed in photoreceptors, strongly impairs the activity of the enzyme. The resulting accumulation of cGMP is believed to be responsible for progressive cell death of photoreceptors, ultimately leading to blindness.

There is some evidence for a role of striatal PDEs in the pathogenesis of neurodegenerative disorders such as Parkinson's disease (PD) and Huntington's disease (HD). These are associated with disturbances of dopaminergic innervation and degeneration of specific neuronal populations in the striatum. Remarkably, there is an apparent correlation between the expression of dopamine receptors, dopaminergic innervation and the expression of some PDEs, with PDE10A as the most fascinating example: PDE10A appears to be highly expressed in GABAergic spiny projection neurons with localization to the membrane of dendrites and dendritic spines (Hebb et al. 2004; Xie et al. 2006). Reminiscent of the picture described for PDE6 in photoreceptors, a decrease in the protein levels of the striatum-enriched PDE10A has been found to precede the actual impairment of motor functions in R6/1 and R6/2 Huntington's disease (HD) mice (Hebb et al. 2004; Hebb and Robertson 2007). Likewise, a significant decrease of PDE10A protein in the caudate-putamen has been observed in patients suffering from HD. In line with these findings, the PDE10A inhibitor papaverine induces distinct motor

perturbations when administered to experimental animals and exacerbates motoric symptoms associated with HD (Siuciak et al. 2006a; Hebb et al. 2008).

Another interesting PDE with an expression pattern in brain similar to that of PDE10A (e.g. in striatal spiny neurons) is the calcium-calmodulin-regulated PDE1B (Furuyama et al. 1994; Polli and Kincaid 1994; Reed et al. 2002). Mice with a genetic ablation of the PDE1B show locomotor dysfunction believed to result from interruption of normal dopaminergic signaling in the striatum (Reed et al. 2002). Similarly, as described for the PDE10A, protein levels of PDE1B have been found to be reduced during early stages of HD in transgenic mice models (Luthi-Carter et al. 2000; Hebb et al. 2004). But unlike PDE10A, there is no obvious correlation between decreased expression of the PDE1B in the striatum and progression of motor dysfunction. In addition, it has to be pointed out that the motoric phenotypes resulting from deficiency of PDE10A and PDE1B are quite opposite: the corresponding knockout mice are characterized by either reduced ($PDE10A^{-/-}$) or enhanced ($PDE1B^{-/-}$) locomotor activity (Reed et al. 2002; Siuciak et al. 2006b) indicating that the two PDEs are involved in distinct intracellular signaling cascades. This fits with the long-standing finding that papaverine unlike most other PDE inhibitors does not potentiate the L-dopa-induced locomotor response (turning) in animal models of PD. In contrast to those, papaverine can even reverse turning induced by L-dopa and atropine (Fredholm et al. 1976).

The phenotype in PDE1B-deficent mice could imply that a loss of PDE1B function results in enhanced activation of striatal signaling pathways, which are shared with the signaling cascade downstream of dopamin receptors. This view is supported by the finding that phosphorylation of the cGK substrate DARPP-32 observed following activation of dopamin D_1 receptors agonist is significantly increased in striatal tissue obtained from PD1EB knockout mice (Reed et al. 2002). These data would suggest inhibition of PDE1B as a possible alternative approach in the treatment of neurodegenerative diseases. As discussed above and in contrast, inhibition of the PDE10A is not expected to be beneficial. In the end, although there is an explicit need in additional options for the treatment of PD or HD, substances modulating striatal PDEs have an uncertain future as potential therapeutics in these clinical situations.

A possible therapeutic effect of PDE4 inhibitors in the treatment of neurodegenerative conditions has been proposed based on the effects of PDE4 inhibition on CREB signaling, BDNF expression and neurogenesis, discussed above. The following findings support this view: PDE4 inhibitors increase the survival of dopaminergic neurons in vitro (Yamashita et al. 1997); an increase of cAMP by PDE4 inhibition improves axon survival, growth, myelin formation and functional recovery after spinal cord injury.

4.4 PDEs in Circadian Rhythm

In mammals, the circadian rhythm is generated and adaptively synchronized to the solar light cycle by a master clock thought to reside in the hypothalamic

suprachiasmatic nuclei (SNC). An important and conserved feature of the nocturnal phase is its regulation by NO/cGMP-dependent signaling via cGK (Eskin et al. 1984; Prosser et al. 1989; Feil et al. 2005). In rats for example, cGK inhibitors cause a delay of wheel-running activity and rhythmic neuronal activity in SCN slice preparations when applied at the end of the subjective night (Weber et al. 1995; Mathur et al. 1996; Tischkau et al. 2003). This implicates a possible modulatory function of cGMP-dependent PDEs. A recent study in hamsters demonstrates expression of the cGMP-specific PDE5 in the SNC (Agostino et al. 2007). Moreover, administration of the PDE5-selective inhibitor sildenafil accelerates reentrainment to the light-dark cycle following 6 h advancement and enhances the responses to a single light pulse during late night (CT18), which induces a phase advance. Given these findings, one could speculate that PDE5 inhibitors may be useful for the treatment of symptoms related to jet lag and shift work.

5 Conclusion

Continuous efforts to identify molecular mechanisms involved in learning and memory, synaptic plasticity and the pathophysiology of CNS disorders have led to the identification of phosphodiesterases (PDEs) as targets for drugs aimed to treat cognitive dysfunction, psychosis and neurodegenerative diseases. There are three major classes of PDEs according to their substrate specificity: cAMP-specific, cGMP-specific and PDEs hydrolyzing both cAMP and cGMP. Data reviewed illustrate the therapeutic potential of the general approach to modulate the concentration of these cyclic nucleotides using selective PDE inhibitors. Inhibitors of the cGMP-specific PDE5 improve memory function, synaptic plasticity, and likely, reentrainment to the dark-light cycle after phase shifts. Their potential in the corresponding clinical indications needs to be evaluated. PDE4 inhibitors could be useful for improving the symptoms of cognitive decline associated with psychiatric diseases and neurodegeneration, but their use is limited by side effects such as nausea and emesis. Recent findings of specific functions of individual subtypes (e.g. PDE4D in depression) suggest the development of subtype-selective PDE4 inhibitors with an improved side effect profile. Inhibition of the striatum-enriched PDE10A has been proposed as a new approach for the treatment of psychosis. The search for selective inhibitors of this enzyme is ongoing.

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Structural and Biochemical Aspects of Tandem GAF Domains

Joachim E. Schultz

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Abstract The GAF domain is a small-molecule-binding-domain (SMBD) identified in >7400 proteins. However, mostly the ligands are unknown. Here we mainly deal with regulatory N-terminal tandem GAF domains, GAF-A and GAF-B, of four mammalian phosphodiesterases (PDEs) and of two cyanobacterial adenylyl cyclases (ACs) which bind cyclic nucleotides. These tandem GAFs are preceded by N-terminal sequences of variable lengths and a function of their own. In mammals, GAF domains are found only in cyclic nucleotide PDEs 2, 5, 6, 10, and 11. cAMP is the ligand for phosphodiesterase 10, cGMP for the others. Two cyanobacterial ACs, CyaB1 and 2, carry regulatory cAMP-binding tandem GAF domains which are similar in sequence to the mammalian ones. These tandem GAF domains have a prominent NKFDE motif which contributes to ligand binding in an as yet unknown manner. Contradicting structures (parallel vs. antiparallel) are available for the tandem GAF domains of PDE 2 and AC CyaB2. In addition, the structures of

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phosphodiesterase 5 and 10 GAF monomers with bound ligands have been solved. In all instances, cyclic nucleotide binding involves specific protein-ligand interactions within a tightly closed binding pocket and minimal solvent exposure of the ligand. The PDE tandem GAF domains can functionally substitute for the tandem of the cyanobacterial AC CyaB1; e.g. cGMP-regulation is grafted onto the AC using tandem GAFs from PDEs 2, 5 and 11. Studies of GAF domain-regulated PDEs are hampered by the identities of regulator and substrate molecules. Using AC CyaB1 as a reporter which uses ATP as a substrate solves this issue and makes the tandem GAF domains of mammalian PDEs available for detailed kinetic and mechanistic studies. In addition, drugs which potentially act on PDE regulatory domains may be assayed with such a novel test system.

Abbreviations

PDE	Phosphodiesterase
AC	Adenylyl cyclase
cAMP	3′, 5′-Cyclic adenosine monophosphate
cGMP	3′, 5′-Cyclic guanosine monophosphate
cNMP	Cyclic nucleotide monophosphate

1 Introduction and Scope

The term GAF domain was introduced in 1997 by Aravind and Ponting, when they compiled protein domains with similar predicted structural motifs in a bioinformatics study (Aravind and Ponting 1997). The acronym GAF was derived from the initials of the first three characterized proteins that contain these domains. They were mammalian c**G**MP-binding phosphodiesterases, the cyanobacterial **A**denylyl cyclases CyaB1 and CyaB2 from *Anabaena sp*., and a **F**ormate-hydrogen-lyase transcription activator from *E. coli* (Zoraghi et al. 2004). Meanwhile, more than 8,500 GAF domains are annotated in the SMART data base (http://smart.embl-heidelberg.de). They are present in more than 7,400 proteins from all kingdoms of life. Looking at the domain organization of these proteins, a huge variety exists. Only in a few instances, GAF domains appear as stand-alone proteins. Mostly, GAF domains are part of multi-domain proteins, which in one way or the other participate in signal transduction processes. The vast majority of proteins contain a single or a tandem GAF domain. Usually, these GAF domains are associated with additional regulatory domains such as PAS domains, various receiver domains (REC domains), HAMP domains, and a variety of sensory output domains, most often histidine kinases. Other frequent output domains are diguanylate cyclases characterized by a GGDEF motif, phosphohydrolases with a conserved HD motif, and ATPases. In a few instances, bacterial proteins exist where three, four, five or even

six GAF domains are arranged consecutively one by one, e.g. in *Synechococcus* or in *Anaeromyxobacter dehalogenans* that has a multi-sensor signal transduction histidine kinase with six GAF domains. This represents the high end of GAF domains present in a single protein entity. It is obvious that the modes of how different GAF domains are integrated into signal transduction proteins are very distinct. Yet, an understanding of GAF domain functions in these complex proteins is virtually non-existent.

For the purpose of this chapter, I will concentrate mainly on proteins that contain tandem GAF domains and that are relevant to the topic of this series in general and this volume in particular. This will limit deliberations to a few examples in which GAF-mediated signaling has been examined more extensively. These are the mammalian PDE families 2, 5, 6, 10 and 11, i.e. five out of the 11 known PDEs families and, in a surprisingly close relationship with these, two cyanobacterial ACs, CyaB1 and CyaB2. All of the aforementioned proteins are dimeric. While for the cyanobacterial ACs, dimerization is a precondition for enzyme activity for PDEs, dimerization appears not to be required for catalytic activity because the isolated monomeric catalytic domain of PDE5 is catalytically active (Fink et al. 1999). These proteins share similarity in their domain organization in that they contain an N-terminal tandem GAF domain linked to a C-terminal output-domain. In addition to GAF domains, the ACs contain a PAS domain of unknown function located between the tandem GAF domain and the cyclase domain. The PAS domain appears to be dispensable for GAF signaling (unpublished data). The N-terminal tandem GAF domains have significant sequence identities and probably share structural similarities as well (for a comprehensive alignment see Fig. 1). In spite of the enormous evolutionary distance of about three billion years between cyanobacteria and humans, the cyanobacterial AC CyaB1 has been successfully used as a reporter enzyme to biochemically assay and characterize mammalian PDE tandem GAF domains.

Among the five PDE families that carry N-terminal tandem GAF domains, the predominantly retinal PDE6 family stands out. Distinct PDE6 isoforms exist for cones and rods with the rod PDE6 being unique as a PDE6α,β heterodimer. Furthermore, low molecular weight inhibitory γ-subunits bind to form a non-activated PDE6α,β,γ_2 heterotetrameric holoenzyme (Mou and Cote 2001). On top of this, two PDE6 rod isoforms exist, which differ as one isoform binds yet another regulatory subunit, a 17 kDa δ-subunit (Mou et al. 1999). Thus it appears that the PDE6 isoforms in cones and rods present themselves as a PDE system set apart from the other similar PDE families with an identical domain organization by peculiar regulatory features. These are necessary in a sensory organ dealing with perception of light, which requires regulation on extremely short timescales. Because of these inherent regulatory complexities that are far from being fully understood, the PDE6 family will not be discussed anywhere further in this chapter with its limited scope and space.

```
hPDE2-A     LQLCGELYDLDASSLQLKVLQYLQQETRASRCCLLLVSEDN----------------------LQL   274
hPDE5-A     VKDISSHLD--VTALCHKIFLHIHGLISADRYSLFLVCEDSSN-------------DKFLISRLFD   206
hPDE6A-A    LLRDFQENLQ-TEKCIFNVMKKLCFLLQADRMSLFMYRTRNG--------------IAELATRLFN   114
hPDE10-A    IEQRLDTGGD-NQLLLYELSSIIKIATKADGFALYFLGECNN------------------SLCIFT   128
hPDE11-A    VKDISNDLD--LTSLSYKILIFVCLMVDADRCSLFLVEGAAAG-------------KKTLVSKFFD   259
cyaB2-A:    SMLDSHGFENILQEMLQSITLKTGELLGADRTTIFLLDEEKQ----------------ELWSIVAA   122
hPDE2-B     AKNLFTHLDD-VSVLLQEIITEARNLSNAEICSVFLLDQN------------------ELVAKVFD   446
hPDE5-B     ASLIFEEQQS-LEVILKKIAATIISFMQVQKCTIFIVDEDCS----------------DSFSSVFH   385
hPDE6A-B    GSKVFEELTD-IERQFHKALYTVRAFLNCDRYSVGLLDMTKQKEFFDVWPVLMGEVPPYSGPRTPD   309
hPDE10-B    SKTYFDNIVA-IDSLLEHIMIYAKNLVNADRCALFQVDHKN----------------KELYSDLFD   305
hPDE11-B    VNDLFEEQTD-LEKIVKKIMHRAQTLLKCERCSVLLLED------------------IESPVVKFT   439
cyaB2-B     VKSLSQSSLD-LEDTLKRVMDEAKELMNADRSTLWLID--------------------RDRHELWT   306

PDE2-A      S-CKVIGDKVLG-----EE----VSFP-------LTGCLGQVVEDKKSIQLK-DLTSEDVQQLQS--- 321
hPDE5-A     VAEGSTLEEVSN-----NC----IRLE------WNKGIVGHVAALGEPLNIK-DAYEDPRFNAEVD-- 256
hPDE6A-A    VHKDAVLEDCLVM--PDQE----IVFP------LDMGIVGHVAHSKKIANVP-NTEEDEHFCDFVD-- 167
hPDE10-A    PPGIKEGKPRLIP---AGP----ITQG--------TTVSAYVAKSRKTLLVE-DILGDERFPRGTG-- 178
hPDE11-A    VHAGTPLLPCSSTE-NSNE----VQVP------WGKGIIGYVGEHGETVNIP-DAYQDRRFNDEID-  313
cyaB2-A     GE-GDRSL---------LE----IRIP------ADKGIAGEVATFKQVVNIPFDFYHDPRSIFAQKQE 169
hPDE2-B     GG------VVDD---ESYE----IRIP------ADQGIAGHVATTGQILNIP-DAYAHPLFYRGVD-- 492
hPDE5-B     MECEELEKSSDTLT-REHD-----ANK------INYMYAQYVKNTMEPLNIP-DVSKDKRFPWTTENT 440
hPDE6A-B    GREINFYKVIDYIL-HGKEDIKVIPNPPPDHWALVSGLPAYVAQNGLICNIM-NAPAEDFFAFQKEPL 375
hPDE10-B    IGEEKEGKPVFK---KTKE----IRFS------IEKGIAGQVARTGEVLNIP-DAYADPRFNREVD-- 357
hPDE11-B    SFELMSPKCSADAENSFKESME---KSSYSDWLINNSIAELVASTGLPVNIS-DAYQDPRFDAEADQI 504
cyaB2-B     KITQDNGS--------TKELRVPIGKGF-------AGI---VAASGQKLNIPFDLYDHPDSAT-AKQI 355

hPDE2-A   --MLGCELQAMLCVPVISRATDQVVALACAFN-KLE-------------------GDLFTDEDEHVIQHCFHYTST  375
hPDE5-A   -QITGYKTQSILCMPIKNHREE-VVGVAQAINKKSGN------------------GGTFTEKDEKDFAAYLAFCGI  312
hPDE6A-A  -ILTEYKTKNILASPIMNGKD--VVAIIMAVN-KVD-------------------GSHFTKRDEEILLKYLNFANL  220
hPDE10-A  -LESGTRIQSVLCLPIVTAIGD-LIGILELYR-HWG-------------------KEAFCLSHQEVATANLAWASV  232
hPDE11-A  -KKTGYKTKSLLCMPIRSSDGE-IIGVAQAIN-KIPE------------------GAPFTEDDEKVMQMYLPFCGI  368
cyaB2-A   -KITGYRTYTMLALPLLS EQGRLVAVVQLLNKLKPYSPPDALLAERID------NQGFTSADEQLFQEFAPSIRL  237
hPDE2-B   -DSTGFRTRNILCFPIKNENQE-VIGVAELVN-KIN-------------------GPWFSKFDEDLATAFSIYCGI  546
hPDE5-B   GNVNQQCIRSLLCTPIKNGKKNKVIGVCQLVN-KMEENTGK--------------VKPFNRNDEQFLEAFVIFCGL  500
hPDE6A-B  -DESGWMIKNVLSMPIVNKKEE-IVGVATFYN-RKD-------------------GKPFDEMDETLMESLTQFLGW  426
hPDE10-B  -LYTGYTTRNILCMPIVSRGS--VIGVVQMVN-KIS-------------------GSAFSKTDENNFKMFAVFCAL  410
hPDE11-B  --KSGFHIRSVLCVPIWN-NH-QIIGVAQVLN-RLD-------------------GKPFDDADQRLFEAFVIFCGL  556
cyaB2-B   DQQNGYRTCSLLCMPVFN-GDQELIGVTQLVN-KKKTGEFPPYNPETWPIAPECFQASFDRNDEEFMEAFNIQAGV  429
```

Fig. 1 Sequence alignment of GAF-A and GAF-B domains of mammalian phosphodiesterases and the cyanobacterial adenylyl cyclase cyab2. Red lettering indicates that the majority of residues at this position are conserved. The high extent of sequence conservation extends as well to the tandem GAF domain from the cyab1 adenylyl cyclase (not shown)

2 Molecular Structure of GAF Domains

2.1 The PDE2 Tandem GAF Structure

The first reported structure of a GAF domain was the YKG9 protein of *Saccharomyces cerevisiae* (Ho et al. 2000). This GAF domain does not bind a cyclic nucleotide as do all tandem GAF domains of mammalian PDEs and lacks a physiological function. Nevertheless, it was misused to model the PDE5 GAF-A domain and to predict individual amino acid residues potentially important for cGMP binding. In view of a PDE2 tandem GAF structure and ensuing mutational studies, these predictions have turned out to be completely irrelevant.

In 2002, a crystal structure of the mouse PDE2 tandem GAF has been solved at 2.9 Å resolution (Martinez et al. 2002a, b). It is a parallel dimer related by a twofold symmetry (Fig. 2a). One cGMP is bound in each of the GAF-B domains, whereas

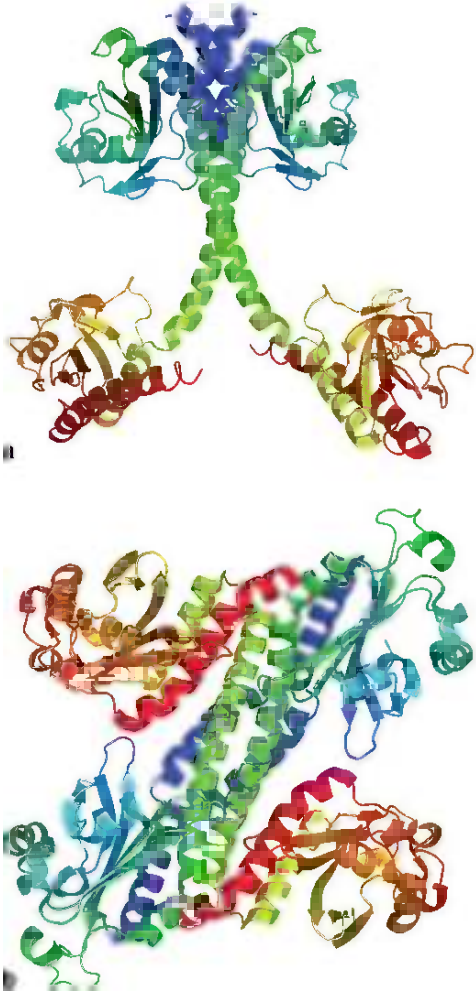

Fig. 2 Structure of the antiparallel PDE2 tandem GAF **a** and the antiparallel cyanobacterial tandem GAF from cyab1 **b** (Martinez et al. 2002b; Martinez et al. 2005). The colouring is from blue, N-terminus, to red, C-terminus and highlights the differences. The bound ligands, cGMP in GAF-B in 2A and cAMP in GAF-A and -B, have been omitted for clarity

the GAF-A domains, albeit of similar structural design, do not contain a ligand. GAF-A and GAF-B are separated by a long connecting α-helix, which is important for dimerization (Fig. 2a).

The cGMP moiety, bound in the GAF-B pocket in its anti-conformation is completely buried in the binding pocket (Martinez et al. 2002). This implies that, to bind or release cGMP the domain must somehow open up and become accessible from the outside. The PDE2 tandem GAF structure visualize several specific contacts between cGMP and amino acid side chains and carbonyl- and amide-groups

of the main chain, respectively (Martinez et al. 2002b). As far as binding specificity of cGMP over the structurally related cAMP is concerned, only the contacts of the pyrimidine ring of the purine moiety need to be considered because otherwise the molecules are structurally identical. Indeed, the major determinant for binding of cGMP preferentially over cAMP (and for cAMP over cGMP in the structure of the CyaB2 – tandem GAF domain; see below) is the N_1/C_6-region of the purine ring. The carboxyl-group of an aspartate side chain forms a 2.6 Å hydrogen-bond with the protonated N_1 of the guanine moiety, which would be impossible with an adenine ring because its N_1 position is not protonated. Further, an amide nitrogen of the main chain forms a 2.8 Å hydrogen-bond with the exocyclic O^6 of the guanine, which is absent in adenine. A similar bond would be impossible with adenine because of a clash with the exocyclic N^6 amino group at this position. Since we do not have a structure of any PDE tandem GAF domain without bound cyclic nucleotide, i.e. an empty structure, one cannot be sure whether the cNMP contacts visible in the resolved structure really are decisive for purine discrimination or whether other residues, which will only be exposed in an empty, non-activating conformation, will have a major role in ligand discrimination. Perhaps, the molecular interactions that are visible in the closed, cyclic nucleotide filled GAF-B structure of PDE2 may be more important for cGMP-binding affinity than for the direct discrimination between guanine and adenine rings.

With the PDE2 tandem GAF structure solved, an earlier structural problem found an unexpected explanation: a sequence alignment of all five PDE tandem GAF domains (and both cyanobacterial AC GAF domains) identified an $NKX_{(5-24)}FX_3DE$ motif, abbreviated NKFDE motif, in at least one or even both GAF domains (Fig. 1). Several mutagenesis experiments affecting this motif resulted in a complete loss of cyclic nucleotide binding and regulation (McAllister-Lucas et al. 1995; Turko et al. 1996; Wu et al. 2004). In fact, among the huge number of annotated GAF domains, the NKFDE motif may potentially be a signature motif for those that bind cNMPs (Bruder et al. 2006) as for the vast majority of GAF domains ligands have yet to be identified. Therefore, it had been expected that the residues of the NKFDE motif somehow participate in ligand binding. This was not the case (Martinez et al. 2002b). Instead, these residues may have a structural function in the formation of the ligand binding pocket as a salt bridge between the lysine and aspartate residues appears to stabilize the immediate vicinity of the cNMP binding cavity. However, without an empty structure, i.e. without bound ligand, it is impossible to fully evaluate the structural role of the NKFDE motif.

2.2 The CyaB2 Tandem GAF Structure

In 2005, Martinez et al. reported the tandem GAF structure of the cyanobacterial AC CyaB2 at 1.9 Å resolution (Martinez et al. 2005). Because of the considerable sequence similarities of the cyanobacterial and the PDE tandem GAF domains (Fig. 1), this structure must be discussed here. There were two major surprises:

(1) the CyaB2 tandem GAF domain crystallized as an antiparallel homodimer in one highly compact asymmetric unit, consistent with the fact that it is a dimer in solution as demonstrated by size-exclusion chromatography and (2) each GAF-A and GAF-B has a bound cAMP resulting in four cAMP moieties per dimer, which are completely buried in an anti-conformation inside the protein as in the PDE2 structure (Martinez et al. 2002b; Martinez et al. 2005). Identical to the PDE2 tandem GAF structure, the two CyaB2 GAF domains are connected by a 32 amino acid long α-helix. This connecting helix is essentially of identical length as that of the PDE2 tandem GAF and, based on sequence alignments, probably in all other PDE tandem GAF domains. Potentially important is that GAF-A and -B of one CyaB2 GAF tandem appear to contact each other via a salt bridge and thus may directly interact in a functionally important manner, a fact which is supported by biochemical data (see below). Considering the parallel and antiparallel structures, the major dimerization surfaces in the CyaB2 structure are differently arranged when compared to those in the PDE2 tandem GAF. In the CyaB2 GAF tandem, they are formed by the N-terminal α-helices, which are folded toward the centre of the dimer, and the linker helices between GAF-A and -B (Fig. 2b). In the PDE2 GAF tandem, the interacting N-terminal α-helices point outwardly and the interacting surface of the linker α-helices appear reduced, compared to CyaB2. The antiparallel conformation of the CyaB2 GAF tandem dimer results in the juxtaposition of GAF-A from one GAF tandem to GAF-B from the other GAF tandem in the dimer (Fig. 2b). This is in contrast to the parallel PDE2 GAF tandem in which the GAF-A domains of each tandem are close to each other and appear to be directly involved in dimerization, whereas the GAF-B domains are apart. The anti-parallel structure of the CyaB2 tandem GAF would functionally be more compatible with the antiparallel arrangement of the dimeric catalytic regions of all class III ACs from which structures are available (Steegborn et al. 2005; Sinha et al. 2005; Tews et al. 2005; Tesmer et al. 1999; Zhang et al. 1997; Sunahara et al. 1997), because it is reasonable to assume that the structure of the CyaB2 holoenzyme also is antiparallel. However, currently it cannot be decided whether the cyclic nucleotide binding tandem GAF domains generally are in a parallel or antiparallel conformation, or whether both structures actually reflect functional conformations in the context of different catalytic output domains, i.e. of PDEs and ACs. Under these circumstances, one would have to invoke different mechanisms and conformational changes for the activation processes of mammalian PDEs and the cyanobacterial class III ACs. This is difficult to imagine considering that the biochemical properties of PDE tandem GAF domains appear to be identical whether linked to a PDE or a cyanobacterial AC (see below). Parallel or anti-parallel, this pressing question can only be solved with the resolution of additional tandem GAF structures and, preferably, structures which comprise the tandem GAF in conjunction with one of its output domains, i.e. a PDE holoenzyme or the CyaB1 AC.

cAMP binding in the CyaB2 GAF-A and -B binding pockets shows that like in the PDE2 tandem GAF, the ligand is not accessible to the solvent (Martinez et al. 2005). In general, binding of cAMP is similar to cGMP binding in the PDE tandem GAF with the obvious exception of the purine part, which is bound via an H-bond

from Arg-291 to the N_1 of cAMP. This residue would not be able to contact the protonated N_1 in the guanine ring. In PDE2 GAF-B, this position is occupied by a non-ligand isoleucine involved in hydrophobic contacts to the guanine ring (Martinez et al. 2002). Thus, with respect to ligand discrimination, the CyaB2 tandem GAF structure has not really contributed novel insights because the problems discussed above for the PDE2 tandem GAF with respect to ligand accessibility and discrimination remain unanswered. Again, an open structure, i.e. without bound cNMP may yield a clue to how adenine and guanine actually are discriminated when they enter the binding cavity, how the protein structure reorganizes to fully enclose the ligand and how this finally leads to activation of a PDE or AC.

Most recently the structure of a PDE10 GAF-B dimer at 2.1 Å resolution has been made accessible (Handa et al. 2008). It contains cAMP bound to each monomer in a fashion comparable to the above structure and a lig-plot of cAMP binding is available. Although valuable as a further contribution to GAF domain structures, it does not really help in the context of tandem GAF domain signaling. Furthermore, albeit the structure is a homodimer, i.e. suggestive of a parallel orientation of a PDE10 GAF tandem dimer, this cannot be deduced with any degree of certainty. Therefore, it is felt that the question whether dimers of PDE tandem GAF domains or respective PDE holoenzymes are parallel or antiparallel, remains as yet unresolved.

Above, I attempted to summarize the available crystal structure information and outlined a number of open questions, which should be addressed in the future. Several additional structural aspects, over the years, have emerged from biochemical experimentation. However, by necessity the data from such studies are less detailed and of a more global nature.

3 The NKFDE Motif in Tandem GAFs

All cyclic nucleotide binding tandem GAFs have a conserved NKFDE motif either in GAF-A (PDE6 and 11), GAF-B (PDE10), or in both GAF domains (PDE2 and 5 and CyaB1 and B2 ACs) (Zoraghi et al. 2004; Zoraghi et al. 2005). Mutations of this motif, predominantly Asp to Ala, either greatly reduce cNMP binding in PDE5 (Ho et al. 2000; McAllister-Lucas et al. 1995; Turko et al. 1996, 1998a, b; Sopory et al. 2003) or abolish it altogether (Wu et al. 2004; Kanacher et al. 2002; Granovsky et al. 1998). This mutational approach has often been used to successfully pinpoint which of the two GAF domains actually is responsible for ligand binding and signaling. In PDE2, GAF-B binds cGMP, whereas GAF-A does not, although the PDE2 GAF-A domain has a canonical NKFDE motif. In PDE5, it is the other way around, GAF-A binds cGMP, whereas GAF-B does not. The data of the binding studies were corroborated using the PDE5 holoenzyme. Enzyme assays indicated that in PDE5 GAF-A is responsible for signaling, although such assays are critical due to the fact that substrate and allosteric regulator are identical, i.e. such experiments require very carefully balanced assay conditions (Rybalkin et al. 2003; Okada and Asakawa 2002).

For PDE10 and 11, cNMP binding assays were not successful due to low ligand affinity and a K_d well above 9 μM was predicted for PDE10A GAF domains based on negative results of cAMP and cGMP binding studies (Soderling et al. 1999; Yuasa et al. 2000).

Taken together, the data imply that the NKFDE motif is required for correct folding of the cNMP binding pocket, yet the presence of this motif alone is not sufficient for cNMP binding and subsequent signaling. Furthermore, it must be repeated that the NKFDE motif is not, as initially suggested (Martinez et al. 2002b), specific for cGMP binding because its presence is also required for cAMP binding or cAMP regulated tandem GAF domains, such as in PDE10 (see below) or the cyanobacterial ACs, CyaB1 and CyaB2 (Martinez et al. 2005; Bruder et al. 2005; Gross-Langenhoff et al. 2006). Thus, the biochemical data are in agreement with the structural insights that the NKFDE motif is not directly involved in cNMP binding but rather has a supportive structural role in formation of the binding cavity. Actually, it would not come as a surprise to realize in the future that this motif is somehow involved in conformational movements, which lead to opening and closing of the binding pocket.

4 Using the Cyanobacterial AC as an Output Domain for Mammalian PDE Tandem GAF Domains

As indicated above, direct studies of regulation of mammalian PDEs via their N-terminal tandem GAF domains have turned out to be rather difficult. Usually, regulation can only be demonstrated under tightly controlled experimental conditions such as preincubation with high cGMP concentrations, rapid dilution, and low substrate concentrations (Rybalkin et al. 2003; Okada and Asakawa 2002). The main reason for these difficulties is the identity of substrate and allosteric regulator such as cGMP in PDE5, which necessarily generates shifting in vitro concentrations. Considering the cAMP-stimulated cyanobacterial adenylyl cyclases, such a situation does not exist. Here, the substrate is ATP and the allosteric regulator is cAMP, the reaction product, and it is easy to define satisfactory experimental conditions, which allow to biochemically characterize GAF domain signaling without mutual interference.

Because of the obvious similarities in the sequences of the cNMP-binding tandem GAF domains and in the regulation of mammalian PDEs and cyanobacterial ACs by cNMPs, a provocative question was whether the regulatory properties of the mammalian tandem GAFs could be functionally grafted onto the bacterial cyclase and this indeed was possible (Kanacher et al. 2002). We functionally replaced the tandem GAF ensemble of the cyanobacterial AC CyaB1 with that from PDE2 (Kanacher et al. 2002). Thus, a cAMP activated cyclase was converted into a cGMP regulated enzyme. In this construct, an evolutionary distance of about 3 billion years was successfully bridged pointing to the evolution of the basic GAF domain design at a very early point. Such functional replacements have meanwhile been successfully repeated for the tandem GAF ensembles of PDE5, PDE10, and PDE11.

In fact, it has permitted to identify cAMP as a ligand for PDE10 and cGMP for PDE11 (Gross-Langenhoff et al. 2006). An interesting question would be whether construction of reverse chimeras that consist of the cyanobacterial tandem GAF and the mammalian PDE catalytic domain may also be accomplished, for example the generation of a cAMP-regulated PDE5 using the CyaB1 tandem GAF and the PDE5 catalytic domain. Such constructs have not yet been reported. In the hypothetic case indicated above, cAMP would be the allosteric ligand, whereas cGMP would be the substrate to be hydrolyzed, i.e. ligand and substrate would constitute different entities. This would facilitate studies on the regulation of PDE5 by a tandem GAF domain.

Meanwhile, chimeric constructs between mammalian tandem GAFs and the CyaB1 AC as a reporter enzyme have been used quite extensively to biochemically characterize PDE tandem GAF domains (Bruder et al. 2006; Kanacher et al. 2002; Gross-Langenhoff et al. 2006; Linder et al. 2007). Although in an indirect way, these studies have yielded novel insights into potential structural interactions. The first issue to be considered, obviously, is whether the biochemical properties of the mammalian tandem GAF domains, which have been elucidated by cGMP binding assays using heterologously expressed tandem and single GAF domains, are mirrored by corresponding biochemical properties of the chimeric constructs. This has been fully established with the tandem GAFs from PDE2 and PDE5, i.e. these chimeras appear to truly reflect the reported signaling attributes of the mammalian PDE tandem GAF domains (Bruder et al. 2006; Kanacher et al. 2002; Linder et al. 2007). In particular, it has been shown that point mutations in the NKFDE motif abolish signaling in the chimeras as would have been expected from the earlier ligand binding studies (Zoraghi et al. 2004, 2005; Wu et al. 2004; Turko et al. 1996, 1998a, b). Thus, the tandem GAF of PDE2 signals via GAF-B and that of PDE5 via GAF-A in the chimera (Bruder et al. 2006; Kanacher et al. 2002). It is often argued that loss of cNMP stimulation in NKFDE mutants may have been due to disruption of folding of the entire protein. This argument, however, disregards the fact that only cNMP activation is lost in such mutants but not AC activity as such. In addition, a mutation in the NKFDE motif in the PDE2 GAF-A does not impair cGMP activation via its GAF-B domain, rejecting the argument of disruption of folding of the entire protein (Kanacher et al. 2002). To the contrary, this data strengthens the argument that basically all domains are folded correctly and are interacting productively.

Consequently, we have established that the ligand for the PDE10 tandem GAF domain is cAMP and that signaling occurs through the GAF-B and not GAF-A domain, as determined by point mutations in the NKFDE motif (Gross-Langenhoff et al. 2006). Interestingly, rebuilding the diverged NKFDE motif in the GAF-A domain from PDE10 did not result in gain of function, i.e. in signaling through GAF-A. Secondly, we have established cGMP as a ligand for the PDE11 tandem GAF domain (Gross-Langenhoff et al. 2006). Earlier, it has been impossible to elucidate the PDE10 or 11 tandem GAF ligands in cNMP binding assays or the respective holoenzymes as the affinities for cAMP and cGMP have turned out to be low (Gross-Langenhoff et al. 2006). This established that the tandem GAF domains of

PDE10 and 11 probably operate in an identical fashion as those of PDE2 and 5 and most likely cause identical structural rearrangements upon activation of PDEs or the CyaB1 AC.

5 A Role for N-termini in Tandem GAF Signaling?

PDE tandem GAF domains of around 300–380 amino acids are located N-terminally and are usually preceded by rather long N-terminal sequences, e.g. 221 amino acids in PDE2, 148 in PDE5, 82 in PDE10, and 197 in PDE11. Do these N-terminals affect or participate in cyclic nucleotide signaling? Using chimeric constructs with CyaB1, this has been examined using the N-termini from PDEs 5, 10, and 11. The answer was not uniform. For the PDE10 N-terminus, the answer is negative since removal of it does not affect cAMP regulation of the attached cyclase. For the PDE 5 and 11 N-termini, the answer is positive (Bruder et al. 2006; Gross-Langenhoff et al. 2008).

5.1 PDE5

The 148 amino acid long N-terminus of PDE5 has a serine at position 102, which upon phosphorylation increases cGMP affinity (Zoraghi et al. 2005; Corbin et al. 2000; Francis et al. 2002; Rybalkin et al. 2002; Thomas et al. 1990). Is the increased cGMP affinity functionally transmitted to the CyaB1 AC output domain? In a phosphomimetic S102D mutant that projects complete phosphorylation, activation of the cyanobacterial AC occurs at significantly lower cGMP concentrations (Bruder et al. 2006). Obviously, the negative charge at Ser-102 shifts the balance between on- and off-conformations of the PDE5 tandem GAF to lower cGMP concentrations. How this is structurally accomplished remains an open question, because speculations about the structure of the PDE5 N-terminus are futile at present. The effect of the Ser-102 phosphorylation can only be reasonably explained by invoking a tight structural coupling between the PDE5 N-terminus and the subsequent tandem GAF. This conclusion is supported by a second line of experiments. Step-wise removal of the N-terminus indicates that up to Ser-102, an N-terminal structure is maintained, which interacts with the tandem GAF in that basal AC activity is maintained at a low level and cGMP affinity is not much affected (Bruder et al. 2006). Removal of 102 and more amino acids from the N-terminus, however, induces a step increase in basal AC activity and, consequently, a reduced capability of cGMP to activate the reporter enzyme (Bruder et al. 2006). Structurally, the data would fit a picture, where the N-terminus and the tandem GAF intimately interact and the compounded complex inhibits AC activity. cGMP-mediated AC activation would then constitute a release from inhibition rather than a straight forward activation. This view is

bolstered by the fact that once inhibition is partially relaxed, e.g. by removal of the N-terminus, the maximal AC activity which can be attained upon cGMP addition, is not shifted upward.

5.2 PDE11

The PDE11 family is composed of four splice variants PDE11A1 to 4. Only PDE11A4 has an unabridged tandem GAF domain and a 196 amino acid long N-terminus. Therefore, it is reasonable to assume that only PDE11A4 can be regulated in a manner comparable to PDEs 2, 5, and 10 because the full tandem GAF is required for interdomain signaling (Bruder et al. 2006). It follows that PDE11A4 is the only isoform suitable to be discussed in this context. The tandem GAF domain of human PDE11A4 requires 72 μM cGMP for a half-maximal activation of the cyanobacterial AC CyaB1, maximal stimulation is restricted to about threefold compared to up to 200-fold in chimeric PDE5 constructs (Bruder et al. 2006; Gross-Langenhoff et al. 2006). Obviously, 72 μM cGMP is difficult to attain intracellularly and the question is how significant is this finding in physiological terms? The PDE11A4 N-terminus has two phosphorylation sites for cNMP-dependent protein kinases (Ser-117, Ser-168). Phosphorylation of both sites by cAMP-dependent protein kinase decreases the EC_{50} for cGMP from 72 to 23 μM. It is most difficult to evaluate whether such in vitro phosphorylations proceed stoichiometrically. Phospho-mimetic point mutations of both serine residues (S117D/S167D), which project complete phosphorylation, lowers the EC_{50} to 16 μM (Gross-Langenhoff et al. 2008) supporting the data from the phosphorylation experiments. Stimulation of PDE11A4 by these cGMP concentrations appears physiologically meaningful. The situation is similar to the PDE5 tandem GAF, where we are dealing with a single serine residue, the phosphorylation of which also increases ligand affinity. Sequence alignments in conjunction with the x-ray structures indicate that 196 amino acids precede the start of the GAF domains in PDE11A4. As has been done with the PDE5 N-terminus, we shortened the PDE11A4 N-terminus step by step and investigated basal and cGMP stimulated CyaB1 AC activity (Gross-Langenhoff et al. 2008). Removal of up to 169 amino acids did not affect basal activity dramatically and cGMP concentrations necessary for half-maximal AC activation were generally increased. However, removal of 176, 186, or 196 amino acids had a dual effect: basal AC activities were increased up to sixfold, i.e. we see a presumed de-inhibition of the CyaB1 AC as outlined for the PDE5 experiments. More importantly, the EC_{50} concentration for cGMP activation dropped to below 10 μM in all constructs truncated by at least 176 amino acids as a construct truncated by 168 amino acids had an EC_{50} of 224 μM. The rather modest 3-fold stimulation by cGMP remained unaffected by the truncation. These findings show again that the N-terminal region may have two separate effects: one that affects cGMP affinity of the tandem GAF domain, more specifically of the GAF-A domain of PDE1A4, and a second component that acts directly on the catalytic activity, possibly in a structural ensemble together with the tandem GAF domain. The data also demonstrate that in order to

gain further molecular insights, it is imperative that an attempt be made to elucidate the structure of PDE N-termini, preferably together with the respective tandem GAF domain, and possibly recognize common structural principles.

5.3 On the Relationship of GAF-A and GAF-B Domains

CyaB1 AC chimeras that contained only one of the tandem GAF domains of PDE5, are unregulated, i.e. for a functional signaling entity both domains of the GAF tandem are needed (Bruder et al. 2006). But what exactly is the structural and functional relationship of the two GAF domains in the tandem? We took advantage of the fact that the tandem GAF of PDE10 uses cAMP as an allosteric ligand and signals via the GAF-B domain (Gross-Langenhoff et al. 2006), and that the tandem GAF of PDE5 uses cGMP and signals via its GAF-A domain (Bruder et al. 2006; Rybalkin et al. 2003). An interesting question is, therefore, whether a chimera that consists of the cGMP-signaling GAF-A from PDE5 and the cAMP-signaling GAF-B domain from PDE10 might be bifunctional, i.e. stimulated equally by both the second messengers, cGMP and cAMP. Treating the N-termini, GAF-A, the linker between GAF-A and -B, and GAF-B as four separate entities, we generated an array of altogether 10 chimeras comprising most possible permutations (Fig. 3). On the one hand, the data were surprising in that a PDE5/PDE10 tandem GAF chimera could be stimulated at all. On the other hand, they were disappointing because the PDE5 GAF-A domain, responsible for cGMP binding and signaling in the PDE5 tandem GAF was not functional in any of the chimeras (Hofbauer et al. 2008). Even in a chimera, which had the N-terminus, GAF-A and the linker region from the PDE5 tandem GAF and only the GAF-B from PDE10, the GAF-A domain of PDE5 was unable to signal to the CyaB1 reporter cyclase (see also below). This strongly argues for a rather specific interaction between the GAF-A and -B domains in the tandem. Taken together, the data suggest that in a tandem GAF domain, a highly ordered and tuned structural interaction exists between GAF-A, the α-helical linker and GAF-B. In addition, in several, yet not all instances secondary modifications of the N-termini may affect signal transduction. So far, the data do not seem to indicate that the N-terminus is directly involved in interactions between GAF-A and -B. However, more experiments are needed to establish this as a general rule. In the anti-parallel structure of the CyaB2 tandem GAF, a salt bridge between GAF-A and -B is recognized that cannot form in the parallel PDE2 tandem GAF structure because of the large distance between GAF-A and -B (Fig. 2) (Martinez et al. 2002b; Martinez et al. 2005). For the correct interaction between GAF-A and -B, the origin of the α-helical linker turned out to be of great significance (Fig. 3). When the N-terminus, GAF-A and the linker are from PDE5 and GAF-B from PDE10, cNMPs do not stimulate AC activity. However, when the N-terminus and GAF-A originated from PDE5 and the linker and GAF-B are from PDE10, signaling via cAMP is observed (Fig. 3). The EC_{50} of 7 μM is identical to the PDE10 tandem GAF linked to the CyaB1 reporter (Gross-Langenhoff et al. 2006; Hofbauer et al. 2008). Even

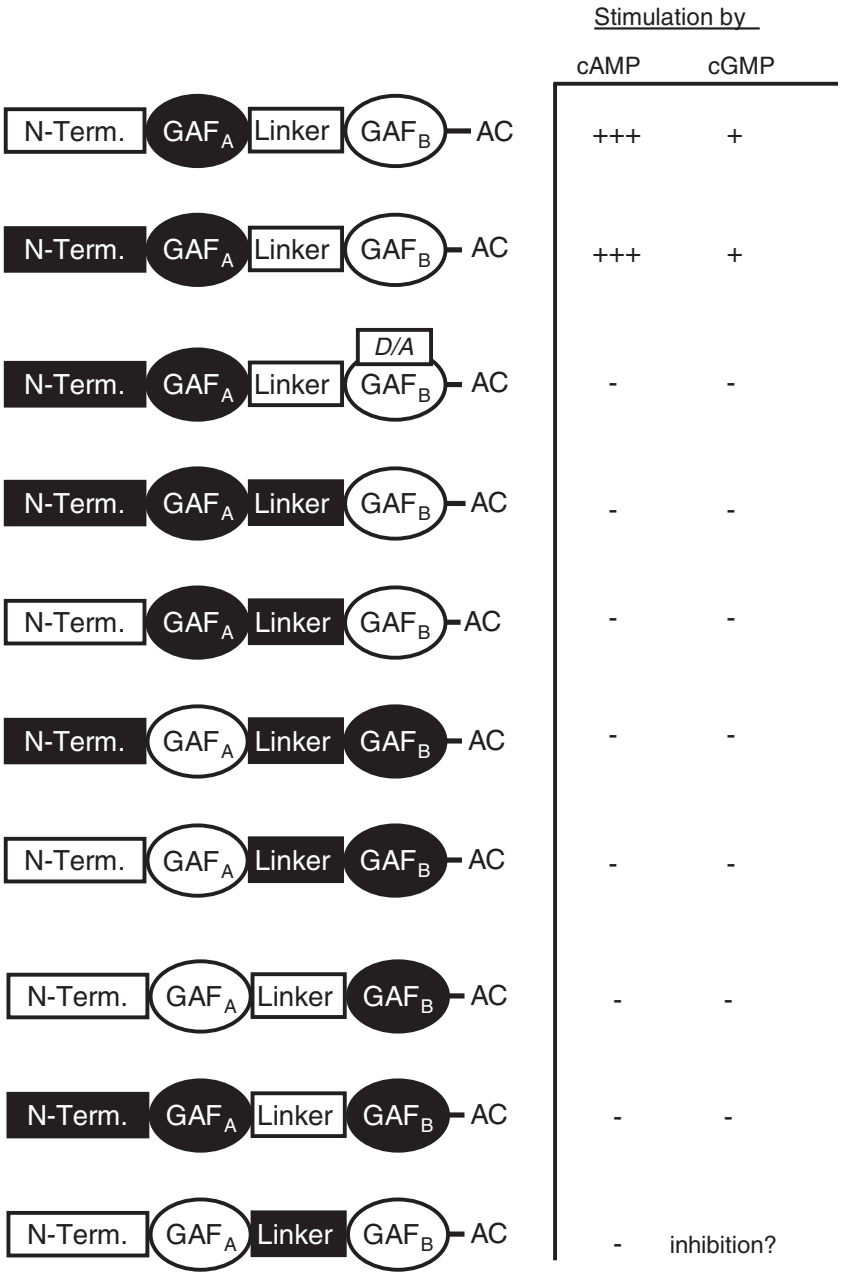

Fig. 3 Schematic drawing of various tandem GAF chimeras which were generated from domains of the PDE5 (labelled black) and 10 (open symbols) tandem GAF domains. The domain boundaries of the regions are: hPDE5A1: N-terminus, aa1-143; GAF-A, 144–310; linker, 311–346; GAF-B, 347–513. HPDE10: N-terminus, 14–72; GAF-A, 73–230; linker, 231–266; GAF-B, 267–422; cyab1 adenylyl cyclase, 386–859. On the right the functionality is indicated qualitatively

the effect of cGMP, which activates to a lesser extent, is mediated exclusively via PDE10 GAF-A. Using a D/A mutant in the NKFDE motif of the PDE10 GAF-B in this construct abolishes any cAMP or cGMP effect (Fig. 3). Two rather unexpected conclusions can be drawn from these observations. First, the function of the PDE5 GAF-A domain in the chimera is obviously reduced to a structural role and the PDE5 GAF-A must be compatible to interact with the PDE10 GAF B in a manner, which preserves the signaling functionality of the latter. Second, swapping the linkers between PDE5 and PDE10 tandem GAF domains abrogated signaling completely in either chimera pointing to highly specific structural interactions between individual GAF domains organized in a tandem ensemble, which is structurally organized by the linker region.

6 Conclusions

Currently, we have two structures of tandem GAF domains that are difficult to reconcile as both being physiologically meaningful. Only the elucidation of further structures of tandem GAFs, e.g. from PDE5, 10, or 11 will help to evaluate what will turn out to be the functionally relevant tandem provided that the assumption is correct that only a single functionally relevant structure exists. The structure of the PDE10 GAF-B dimer with bound cAMP is problematic and of no help in this respect, because it only presents a functionally irrelevant partial structure. To date, we have been unable to observe signaling in chimeras, which only had a single GAF domain and all evidence indicates that for proper signaling, a tandem arrangement is required in all respective PDEs and the cyanobacterial ACs (Bruder et al. 2006). All biochemical work done in conjunction with a cyanobacterial reporter AC assumes that signaling in this chimera is identical or at least very similar to signaling in mammalian PDEs. Although current evidence certainly supports this claim, one should not completely lose sight of the artificial nature of these chimeras. Nevertheless, the unexpected functionality of these chimeras opened the way to biochemically characterize several features of how individual GAF domains must interact in a dimer to be productive. Further, in these constructs the tandem GAF domains of mammalian PDEs can be examined as drug targets independent from the catalytic PDE region using the cyanobacterial AC CyaB1 as a reporter enyzme.

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Most recently, a parallel PDE2-holoenzyme structure has been solved (J. Pandit, personal communication).

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Cyclic Nucleotide-Gated Channels

Martin Biel and Stylianos Michalakis

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Abstract Cyclic nucleotide-gated (CNG) channels are ion channels which are activated by the binding of cGMP or cAMP. The channels are important cellular switches which transduce changes in intracellular concentrations of cyclic nucleotides into changes of the membrane potential and the Ca^{2+} concentration. CNG channels play a central role in the signal transduction pathways of vision and olfaction. Structurally, the channels belong to the superfamily of pore-loop cation channels. They share a common domain structure with hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and Eag-like K^+ channels. In this chapter, we give an overview on the molecular properties of CNG channels and describe the signal transduction pathways these channels are involved in. We will also summarize recent insights into the physiological and pathophysiological role of CNG channel proteins that have emerged from the analysis of CNG channel-deficient mouse models and human channelopathies.

Keywords: CNG · Cyclic nucleotide-gated channel · Channelopathies · Knockout · cGMP · cAMP

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1 Introduction

Cyclic nucleotides regulate the opening of several classes of cation channels by binding to a cyclic nucleotide-binding domain (CNBD) that has been conserved across a wide range of proteins, including bacterial transcription factors (e.g. CAP; (Weber et al. 1987)), cAMP- and cGMP-dependent protein kinases (Pfeifer et al. 1999; Taylor et al. 2005) and cAMP-dependent guanine nucleotide exchange factors (Epac; (Bos 2006)). Structurally, cyclic nucleotide-regulated channels belong to the superfamily of pore-loop cation channels (Yu et al. 2005). In vertebrates, three subfamilies of CNBD-containing ion channels have been identified: the CNG, the HCN and the Eag-like K^+ channels (Craven and Zagotta 2006; Hofmann et al. 2005; Kaupp and Seifert 2002). While these channels display the same principal architecture (see below) they differ from each other in terms of their ion selectivity and their activation mode. CNG (cyclic nucleotide-gated) channels pass Na^+, K^+ and Ca^{2+} (Kaupp and Seifert 2002). The channels are strictly ligand-gated because their opening requires the binding of cGMP or cAMP. CNG channels are not restricted to vertebrates but have been also found in several non-vertebrate species including insect species and *C. elegans*. In contrast to CNG channels, HCN (hyperpolarization-activated cyclic nucleotide-gated) channels are principally operated by voltage. In typical members of this ion channel family binding of cAMP or cGMP facilitates opening by shifting the voltage dependence of activation to more positive values. Notably, one particular member of the family, HCN3 seems to be unaffected or even inhibited by cAMP (Mistrik et al. 2005; Stieber et al. 2005). HCN channels carry Na^+ and K^+, while Ca^{2+} is only weakly, if at all, passed (Craven and Zagotta 2006; Hofmann et al. 2005). Like CNG channels HCN channels have been identified in several invertebrates (Galindo et al. 2005; Gauss et al. 1998; Gisselmann et al. 2005). The functional role of the CNBD in voltage-gated Eag-like K^+ channels and plant K^+ channels remains elusive. The effects of cyclic nucleotides on these channels are weak or disputed (Brüggemann et al. 1993; Cui et al. 2001; Hoshi 1995). Cyclic nucleotide-regulated potassium channels have been also identified in sea urchin (Galindo et al. 2007; Strünker et al. 2006) and even in a number of prokaryotes (Clayton et al. 2004). Based on the latter finding one may speculate that an ancestral cyclic nucleotide-regulated cation channel emerged early in evolution by the fusion of a gene encoding a K^+ selective pore-loop channel with a gene encoding a CNBD. The resulting primordial cyclic nucleotide-regulated K^+ channel then evolved to the diverse classes of modern cyclic nucleotide-regulated channels.

In this review, we will focus on CNG channels since only for these channels the physiological significance of cGMP as activating agent has been clearly established in mammalian cells. By contrast, while heterologously expressed HCN channels are sensitive to cGMP, cGMP-dependent regulation of HCN currents has been demonstrated only in a few types of native neurons (Ingram and Williams 1996; Pape and Mager 1992). Thus, it remains open whether cGMP is a regulator of HCN channels under in vivo conditions and, if so, which role such a regulation could fulfill in a physiological setting.

CNG channels have been originally identified in photoreceptors and olfactory receptor neurons (ORNs). In these cells, the channels play a fundamental role in sensory transduction. In addition, CNG channels have been also reported to exist in several other cell types and tissues including brain, kidney, endocrine tissues and sperm cells (Kaupp and Seifert 2002). Despite numerous efforts over the last 15 years, the functional role of the channels in these tissues could not be clarified. In most of the mentioned tissues and cells (with the exception of sperm; refs. (Weyand et al. 1994; Wiesner et al. 1998)) CNG channels were only identified on the level of their respective mRNAs, while protein levels usually were too low to be detected. Moreover, analysis of CNG channel knockout mice did not provide convincing evidence for functions outside the sensory systems. Given the questionability of CNG channel expression in non-sensory systems, we will concentrate here on vision and olfaction, where the physiological relevance of CNG channels is undoubted. We will start with a brief outline of the sensory transduction pathways underlying vision and olfaction (for a more detailed description see (Burns and Baylor 2001; Firestein 2001; Fu and Yau 2007; Kaupp and Seifert 2002; Ma 2007)).

2 Signal Transduction in Photoreceptors

Vision in vertebrates is conferred by the concerted action of two phototransduction pathways, the rod and cone photoreceptor system. Rods are responsible for vision at low light intensity (e.g. twilight), whereas vision at higher light intensity (e.g. daylight) is provided by cones. The cone system also confers color vision since cones exist in two (short- and medium-wave sensitive in most vertebrates) or three variants (short-, medium- and long-wave sensitive in humans and simian primates) with different spectral sensitivities (Ahnelt and Kolb 2000; Yokoyama 2000). In both rods and cones, signal transduction is conferred by the second messenger cGMP that controls the activity of a CNG channel present in the plasma membrane of the outer segments of these cells (Fig. 1). In the dark, the CNG channel is maintained in the open state by a high concentration of cGMP produced by two types of transmembrane guanylyl cyclases (GC-E and F, (Pugh et al. 1997; Yang et al. 1995)). The resulting influx of Na^+ and Ca^{2+} ("dark current") depolarizes the photoreceptor and promotes synaptic glutamate release. Ca^{2+} is not only a permeating ion of CNG channels, it also lowers cGMP sensitivity and, hence, activity of these channels by interacting with calmodulin (rod) or with other not yet characterized Ca^{2+}-binding proteins (Rebrik and Korenbrot 1998). While Ca^{2+}-dependent inhibition of CNG channels is substantial in cones, it is only weak in rod photoreceptors and may be of minor physiological importance in these cells (Fain et al. 2001). Light absorption by opsins initiates a G protein-mediated signaling cascade leading to activation of a cGMP phosphodiesterase, hydrolysis of cGMP and closure of the CNG channel. As a result, the photoreceptor hyperpolarizes and shuts off synaptic transmission. CNG channels provide the only source for Ca^{2+} influx into rod and cone outer segments (Hodgkin et al. 1985; Kaupp and Seifert 2002; Yau and Nakatani 1984). Ca^{2+} entry

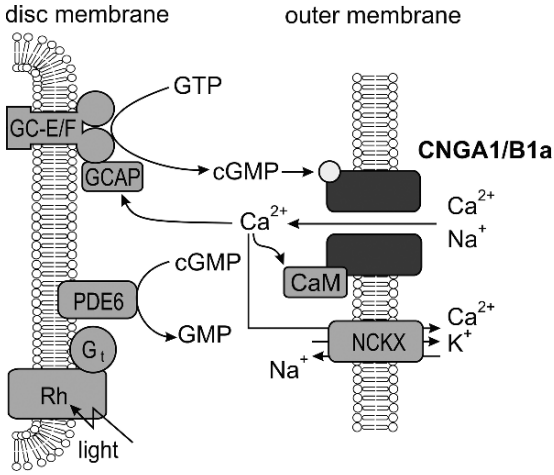

Fig. 1 Phototransduction in rod outer segment. In the dark, the CNG channel (CNGA1/B1a) of the outer membrane is kept open by high concentrations of cGMP produced by guanylyl cyclases E and F (GC-E/F) present in the disc membrane. The resulting influx of Na^+ and Ca^{2+} depolarizes the plasma membrane. Light activates rhodopsin (Rh) which in turn activates transducin (G_t) whose α subunit activates a phosphodiesterase (PDE6) leading to hydrolysis of cGMP. The drop in the cGMP concentration leads to the closure of the CNG channel resulting in membrane hyperpolarization. Ca^{2+} is an important regulator of phototransduction. At high concentrations Ca^{2+} binds to guanylyl cyclase-activating proteins (GCAP) leading to an inhibition of guanylyl cyclases. High levels of Ca^{2+} also lead to a slight reduction of the cGMP affinity of the CNG channel via Ca^{2+} calmodulin (CaM)-mediated feedback inhibition. Ca^{2+} is cleared from the outer segment via a Na^+-Ca^{2+}-K^+-exchanger (NCKX). At low Ca^{2+} levels GCAPs switch to the Ca^{2+}-free form that is an activator of GCs

is balanced by Ca^{2+} extrusion through the activity of a Na^+/Ca^{2+}-K^+ exchanger (Cervetto et al. 1989; Hodgkin et al. 1985; Schnetkamp 2004; Yau and Nakatani 1985). Thus, light also decreases the cellular Ca^{2+} concentration because it shuts down Ca^{2+} entry while the Na^+/Ca^{2+}-K^+ exchanger continues to clear Ca^{2+} from the cytosol. The low Ca^{2+} concentration contributes to the recovery from light response which is achieved by several processes including, inactivation of opsin and phosphodiesterase, and restoration of cGMP levels by activation of the guanylyl cyclase via guanylyl cyclase activating proteins (GCAP1–3; (Palczewski et al. 2004)).

3 Signal Transduction in Olfactory Neurons

Phototransduction in mammalian retina uniformly relies on the cGMP/CNG channel-mediated pathway described above. In contrast, olfactory transduction is significantly more diverse. Multiple chemosensory systems have been identified in the main olfactory epithelium (MOE), the vomeronasal organ (VNO), the septal organ (SO) and the Grueneberg ganglion (GG) of the nose (Ma 2007). While

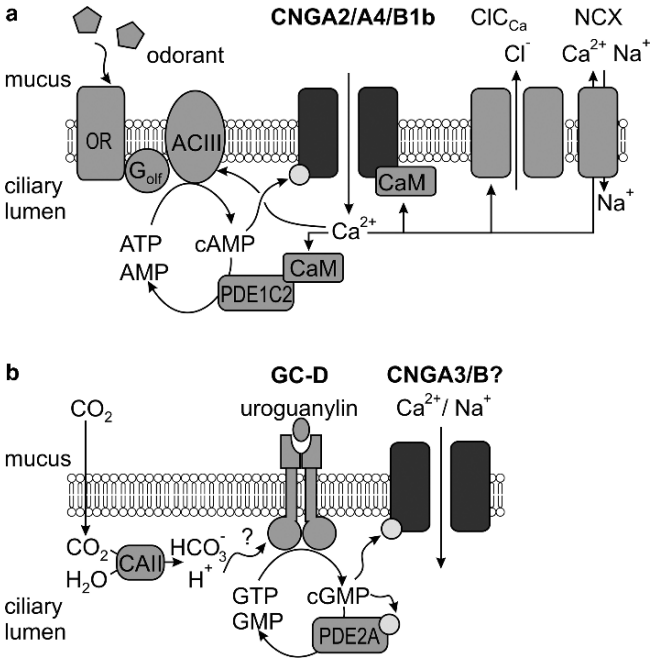

Fig. 2 Olfactory transduction in cilia of olfactory receptor neurons. **a** cAMP-mediated transduction. Odorants bind to odorant receptors (OR) activating a G-protein (G_{olf}) that in turn stimulates the adenylyl cyclase type III (ACIII). The rise in cAMP opens the CNG channel which passes a depolarizing Na^+ and Ca^{2+} current. Ca^{2+} opens a Ca^{2+}-activated Cl^- channel (ClC_{Ca}) leading to Cl^- efflux which further depolarizes the cell. Ca^{2+} binds to calmodulin lowering the cAMP sensitivity of the CNG channel and activating a phosphodiesterase (PDE1C2). Ca^{2+} also reduces the activity of ACIII via CaM-kinase II-mediated phosphorylation. Ca^{2+} is extruded by a Na^+-Ca^{2+} exchanger (NCX). **b** cGMP-mediated pathway. Cyclic GMP produced by the guanylyl cyclase D (GC-D) activates a CNG channel leading to influx of Ca^{2+} and Na^+ and a subsequent membrane depolarization. The CNG channel contains the CNGA3 subunit and may also contain B subunits. GC-D is activated by (uro)guanylin probably via direct binding. In addition, activity of GC-D is enhanced by the carboanhydrase II that transforms CO_2 and H_2O into HCO_3^- and H^+. Hydrolysis of cGMP is conferred by a cGMP-regulated phosphodiesterase (PDE2A)

chemotransduction in the VNO does not seem to involve CNG channel activity, the vast majority of olfactory receptor neurons (ORNs) of the MOE, as well as those of the SO employ CNG channels for sensory transduction. In the MOE, two principal populations of CNG channel expressing ORNs coexist, the major one using cAMP (Fig. 2a) and a minor one utilizing cGMP for sensory transduction (Fig. 2b). In both types of neurons, olfactory transduction takes place in the cilia. Cilia are thin protrusions of the dendritic end of ORNs which harbors all molecular components required for chemoelectrical transduction. They represent the functional equivalent of the outer segments of photoreceptors. In the major fraction of ORNs binding of an odorant to its cognate receptor (Buck and Axel 1991) leads to activation of a ciliary adenylyl cyclase (Bakalyar and Reed 1990; Wong et al.

2000). The ensuing rise in the cAMP level opens the olfactory CNG channel that conducts mainly Ca^{2+} and little Na^{+} under physiological ionic conditions (Frings et al. 1995; Nakamura and Gold 1987). The resulting influx of Ca^{2+} slightly depolarizes the membrane and, at the same time, it activates a Ca^{2+}-activated Cl^{-} channel (Kleene and Gesteland 1991; Kurahashi and Yau 1993; Lowe and Gold 1993). It was suggested that bestrophin-2 is a molecular component of the olfactory Ca^{2+}-activated Cl^{-} channel (Pifferi et al. 2006b) and that gating of this channel involves calmodulin (Kaneko et al. 2006). The intracellular Cl^{-} concentration in ORNs is maintained above electrochemical equilibrium by a Na^{+}-$2Cl^{-}$-K^{+} co-transporter (Kaneko et al. 2004; Reisert et al. 2005). Thus, activation of the Cl^{-} channel results in Cl^{-} efflux and strong membrane depolarization and acts as efficient amplifier of the primary CNG current (Kleene and Gesteland 1991; Kurahashi and Yau 1993; Lowe and Gold 1993). Ca^{2+} also serves as negative feedback signal and modulator of signal transduction (Matthews and Reisert 2003). Fast odor adaptation is achieved by reduction of the ligand sensitivity of the olfactory CNG channel through a Ca^{2+}-calmodulin (CaM)-dependent mechanism (Kurahashi and Menini 1997). Unlike in rod photoreceptors, CaM-mediated feedback inhibition of the CNG channel is very pronounced and was long believed to play the dominant role in fast odor adaptation (Pifferi et al. 2006a). However, recent data by (Song et al. 2008) suggest that this fast CaM-mediated feedback inhibition of the channel is less important than previously thought and functions primarily to terminate ORN responses. Heterologously expressed olfactory CNG channels were shown to be also inhibited by phosphatidylinositol-3,4,5-trisphosphate (PIP_3) via a direct interaction (Brady et al. 2006). PIP_3 occludes CaM binding and blocks the feedback inhibition by this protein. It was speculated that PIP_3-mediated CNG channel inhibition potentially may serve to reduce overall olfactory sensitivity in the presence of complex odors (Brady et al. 2006; Spehr et al. 2002). In addition to inhibiting CNG channel activity, Ca^{2+} also exerts an inhibitory effect on the ciliary adenylyl cyclase (Leinders-Zufall et al. 1999; Wei et al. 1998) and stimulates cAMP hydrolysis by a CaM-dependent ciliary isoform of the phosphodiesterase (PDE1C2) (Boccaccio et al. 2006; Borisy et al. 1992; Yan et al. 1995). Both processes contribute to slow odor adaptation. Odorant molecules might also contribute to adaptation by directly inhibiting the olfactory CNG channel (Chen et al. 2006).

As pointed out above, a minor subset of ciliated ORNs in the MOE utilizes cGMP as principal second messenger (Zufall and Munger 2001). These neurons were shown to contain the cone-type CNG channel (Meyer et al. 2000), a membrane-bound guanylyl cyclase (GC-D, (Fulle et al. 1995)), and a cGMP-stimulated phosphodiesterase (Juilfs et al. 1997). In contrast, they lack the components typical of other ORNs (Juilfs et al. 1997; Meyer et al. 2000). The GC-D ORNs project to a group of atypical glomeruli in the main olfactory bulb designated as "necklace" glomeruli (Shinoda et al. 1989). A recent study using mice deficient for either GC-D or the cone type CNG channel (CNGA3) revealed that GC-D ORNs recognize the natriuretic peptide hormones uroguanylin and guanylin (Leinders-Zufall et al. 2007). The physiological relevance of this finding has to be determined but it was speculated that GC-D ORNs contribute to the maintenance of salt and water

homeostasis or the detection of cues related to hunger, satiety or thirst. Unlike in photoreceptors where a sensory stimulus (light) leads to hydrolysis of cGMP and subsequent membrane hyperpolarization, the cGMP-cascade in GC-D neurons is excitatory leading to membrane depolarization. It is not yet clear by which signaling pathway peptides stimulate cGMP synthesis in GC-D neurons. In other cell types (e.g. cardiomyocytes, vascular endothelial cells, renal epithelial cells) transmembrane guanylyl cyclases have been shown to be directly activated by binding of natriuretic peptides (e.g. ANP, BNP). Thus, it is tempting to assume that GC-D itself is the primary (uro)guanylin receptor transducing peptide binding into cGMP synthesis (Fig. 2b). In addition, GC-D may be stimulated by the activity of carboanhydrase II which catalyzes the reversible reaction of $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$. It was proposed that the olfactory subsystem comprising GC-D ORNs enables mice to detect CO_2 at near-atmospheric concentrations. (Fig. 2b; (Hu et al. 2007)).

4 Structure and Basic Properties of CNG Channels

CNG channels form a distinct branch within the superfamily of pore-loop channels (Hofmann et al. 2005; Yu et al. 2005). The family of mammalian CNG channels comprises six homologous members, which are classified as A subunits (CNGA1–4) and B subunits (CNGB1 and CNGB3) (Fig. 3a). A and B subunits are also found in

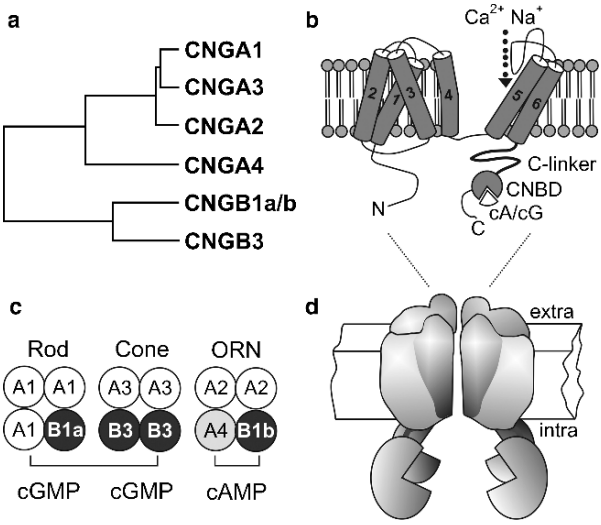

Fig. 3 **a** Phylogenetic tree of mammalian CNG channel subunits. **b** Membrane topology of CNG channel subunits. 1–6, transmembrane segment 1–6; C, carboxy-terminus; CNBD, cyclic nucleotide-binding domain; N, amino-terminus. **c** Subunit composition of the CNG channels from rods, cones and olfactory receptor neurons. The physiological ligand of the two photoreceptor channels is cGMP while the olfactory channel is activated in vivo by cAMP. **d** Model of a tetrameric CNG channel complex

the genome of several invertebrates (e.g. C. elegans and D. melanogaster) suggesting an early segregation of these two channel subfamilies in evolution. Diversity of mammalian CNG channels is further increased by splicing (overview in (Kaupp and Seifert 2002)). Importantly, splicing variants of CNGB1 containing different cytosolic N-termini are specifically expressed in rod photoreceptors (CNGB1a) and ORNs (CNGB1b), respectively (Bönigk et al. 1999; Chen et al. 1993; Körschen et al. 1995; Sautter et al. 1998). A subunits and B subunits share the same principal membrane topology which is characterized by six transmembrane domains (S1-S6), a reentrant pore (P) loop between S5 and S6, and cytosolic N- and C-termini (Fig. 3b). Like the related voltage-gated K^+ channel whose crystal structure has been recently solved (Long et al. 2005), CNG channels are tetramers with the four subunits arranged around the centrally located pore (Higgins et al. 2002). The ion conducting pore is lined by the P-loops and the S6 segments of the four subunits. Three domains are involved in the activation process of CNG channels: (1) the channel gate which is localized in the distal portion of the S6 segment (Giorgetti et al. 2005; Jiang et al. 2002), (2) the CNBD (Kaupp et al. 1989; Zagotta et al. 2003) and (3) the C-linker, a domain that allosterically couples cyclic nucleotide-binding to the channel gate (Paoletti et al. 1999; Sunderman and Zagotta 1999; Zong et al. 1998).

Although all six CNG channel subunits share significant sequence homology only CNGA1, CNGA2 and CNGA3 can be functionally expressed as homomers in heterologous cell systems. However, the biophysical and pharmacological properties of the respective homomeric channels differ in several aspects (e.g. single channel current characteristics, affinity for cyclic nucleotides, permeation properties, regulation by Ca^{2+}) from those of native CNG channels (for detailed review see (Kaupp and Seifert 2002; Matulef and Zagotta 2003)). This discrepancy could be solved in co-expression studies showing that distinct combinations of A subunits and B subunits generated channels that almost perfectly matched the functional properties of native channels. This finding strongly suggested that native CNG channels are heterotetramers. This hypothesis has been confirmed by recent biochemical and FRET studies. Using these methods the subunit compositions and stoichiometries have been determined for the channels expressed in rod photoreceptors (3 CNGA1: 1 CNGB1a) (Weitz et al. 2002; Zheng et al. 2002; Zhong et al. 2002), cone photoreceptors (2 CNGA3: 2 CNGB3) (Peng et al. 2004) and olfactory neurons (2 CNGA2: 1 CNGA4: 1 CNGB1b) (Zheng and Zagotta 2004) (Figs. 3c and d). It should be noted that the stoichiometry of the cone and olfactory channel has only been determined in heterologous expression systems. Since knockout studies suggest that subunit assembly may differ between native and heterologous cells (see below) it will be necessary to confirm the results in purified native channels. Moreover, it remains to be determined whether homomeric CNG channels and CNG channels with subunit compositions different from those of photoreceptors and ORNs exist in other cell types.

CNG channels characterized so far share some common features. All pass Na^+ and K^+, but do not discriminate between them. Calcium is also permeable but at the same time acts as a voltage-dependent blocker of monovalent cation permeability (Dzeja et al. 1999; Frings et al. 1995). Moreover, CNG channels do not desensi-

tize or inactivate when exposed to cyclic nucleotides. They are, however, subjected to feedback regulation via CaM-mediated mechanisms (Bradley et al. 2005; Pifferi et al. 2006a). In the rod photoreceptor, channel inhibition is conferred by binding of CaM to an IQ-type binding site located at the N-terminus of the CNGB1a subunit (Bradley et al. 2004; Weitz et al. 1998). In the olfactory CNG channel, CaM-mediated regulation is more complex. It requires two IQ-type binding sites, one site on the CNGB1b subunit being identical with that present in the rod CNGB1a channel and an additional IQ-type site present in the C-linker of CNGA4 (Bradley et al. 2004). Deletion of either site abolishes fast inhibition by CaM. The nature of the functional interaction between the two CaM binding sites remains to be elucidated.

5 CNG Channel Activation

The opening of CNG channels comprises two principal steps, the ligand binding to the CNBD when the channel is in its closed state and an allosteric conformational change, coupled to ligand binding that opens the channel pore. A quantitative measure of the first step is the ligand affinity while the ligand efficacy (defined as the ratio of the open probabilities at saturating concentrations of cAMP and cGMP) refers to the second step. The recent determination of the molecular structure of the C-linker and the CNBD of HCN channels in conjunction with molecular dynamics simulations, and advanced biophysical and FRET approaches have provided important insights into the molecular correlates of the complex activation process (Flynn et al. 2007; Taraska and Zagotta 2007; Zagotta et al. 2003; Zhou and Siegelbaum 2007). The CNBD shows a highly conserved fold, consisting of an initial α helix (A helix), followed by an eight-stranded antiparallel β roll (β1-β8), a short B helix and a long C helix. The C-linker consists of six α helices (A′-F′). The binding pocket for cyclic nucleotides is formed by a number of residues at the interface between the β roll and the C helix. Subsequent to cyclic nucleotide-binding, four C-linker-CNBD units assemble to a tetrameric gating ring whose intersubunit contacts are largely mediated by the C-linker. Tetramerization is then allosterically coupled via a centripetal movement to the channel gate (Taraska and Zagotta 2007). In the CNBD, cGMP is held in the *syn* configuration while cAMP binds in the *anti* configuration. A threonine residue present in the β7 strand of HCN channels and all six CNG channel subunits (T560 in bovine CNGA1) preferentially stabilizes *syn* cGMP by forming an additional H bond with the guanine ring of cGMP compared to its interaction with the adenine ring of *anti* cAMP (Altenhofen et al. 1991; Zagotta et al. 2003). The opening conformational step involves the movement of the C helix relative to the β roll. For cGMP but not for cAMP, an acidic residue in the C helix (D604 in bovine CNGA1) stabilizes this rearrangement, and promotes channel opening (Flynn et al. 2007; Varnum et al. 1995). Although this mechanism is consistent with the observation that CNG channels select cGMP over cAMP, it does not explain why cAMP is a full agonist of CNGA2 but only a partial agonist of CNGA1 or CNGA3 (Kaupp and Seifert 2002). To explain this difference it was assumed that

the opening conformational change in CNGA1 and CNGA3 is *per se* more energetically costly than that in CNGA2. Thus, full activation of CNGA1 and CNGA3 channels obligatorily requires stabilization of the cyclic nucleotide by both the threonine of the β7 strand and the acidic residue of the C-helix (Gordon and Zagotta 1995). Since this structural precondition is fulfilled for cGMP, this cyclic nucleotide is a full agonist of all CNG channels. In contrast, because it binds to the CNBD in its a*nti* conformation cAMP is not sufficiently stabilized by the C-helix and, hence, does not provide enough conformational energy to fully open CNGA1 and CNGA3. However, the binding energy provided by *anti* cAMP is high enough to activate fully the "easy-to-open" CNGA2 channel. The N-terminal domain including the first two transmembrane segments, may account for the differences in free energy of gating between the different CNG channel types (Tibbs et al. 1997). It also must be considered that, full activation CNGA1 and CNGA3 tetrameric CNG channel complex is achieved by the binding of only two ligand molecules (Biskup et al. 2007). This observation raises the question which constraints partial occupation of cGMP-binding sites plays on the formation and structure of the gating ring. Finally, it is important to note that crystal structures have been only solved for homotetrameric (i.e. symmetrical) C-linker-CNBD complexes while native CNG channels are asymmetrical complexes. It is unlikely that the principles of gating depend on the subunit composition; nevertheless, the fine-tuning of this allosteric process which determines the exact values of ligand affinities and efficacies is probably sensitive to subtle changes in quaternary structure of the channel.

6 CNG Channel Knockout Models and Human Channelopathies

Recent gene knockout studies in mice as well as the analysis of diseases resulting from mutations in human CNG channel genes (CNG channelopathies) have dramatically increased our knowledge on the physiological role and significance of A subunits and B subunits. In the following sections we will summarize the results of these studies. We will focus on vision and olfaction but will also shortly discuss other physiological systems CNG channels were found to be involved in.

6.1 Rod Photoreceptor CNG Channel

6.1.1 CNGA1

Mutations in the CNGA1 gene have been found in patients suffering from retinitis pigmentosa (RP) (Dryja et al. 1995). RP comprises a genetically diverse group of progressive degenerative diseases effecting the photoreceptors of the retina (Kennan et al. 2005). The most common symptoms of RP includes night blindness,

progressive concentric reduction of the visual field and abnormal accumulation of pigmentation in the retina (Kalloniatis and Fletcher 2004). In most cases RP finally leads to legal blindness. At the cellular level the disease is characterized by a primary impairment or total loss of rod function and structure followed by a secondary degeneration of the cones. So far, RP has been mapped to >30 genes (http://www.sph.uth.tmc.edu/Retnet) encoding proteins involved in the visual transduction pathway (e.g. rhodopsin) or required for the maintenance of rod architecture (e.g. peripherin). Mutations in CNGA1 lead to an autosomal recessive form of RP that accounts for about 1% of cases of RP (Dryja et al. 1995). Three of the five mutant alleles identified are null mutants (E76Stop, K139Stop, and deletion of most or the entire CNGA1 transcription unit) because they would encode proteins that are lacking essential channel domains. The remaining two other alleles (S316F and R654DStop) encode channels that fail to reach the plasma membrane in heterologous expression systems. Data of Mallouk et al. (2002) in Xenopus oocytes suggest that the KLKQDStop sequence present at the C-terminus of the R654DStop mutant represents an ER retention signal that effectively prevents CNGA1 targeting to the plasma membrane. Based on experiments in the same system Trudeau et al. proposed an alternative model (Trudeau and Zagotta 2002). These authors claim that truncation of the last 32 amino acids in the R654DStop mutant exposes a normally buried region from the CNGB1a N-terminal domain harboring a retention signal, and, as a consequence, prevents membrane expression of the heteromeric channel.

Up to date, mice lacking the CNGA1 subunit are not available. However, transgenic mice revealing a reduction of about 50% in CNGA1 transcript levels due to the overexpression of a CNGA1 antisense mRNA have been produced (Leconte and Barnstable 2000). These mice show some histological features reminiscent of RP (e.g. reduced number of photoreceptors, apoptotic death of retinal cells). However, electroretinograms (ERGs) of these mice have not been reported so far. Thus, it remains an open issue to which extent the down-regulation of CNGA1 affects rod- and cone-mediated vision. Moreover, it cannot be excluded that the overexpression of the antisense mRNA exerts non-specific effects (e.g. toxic effects caused by suppression of other mRNAs) that may affect the phenotype.

Taken together the genetic data imply that photoreceptors degenerate in response to the absence (or down-regulation) of functional CNG channels in outer segments. The molecular pathways leading to the initiation of cell death in CNGA1-deficient rods are unknown. One possible mechanism refers to the "equivalent-light" hypothesis (Lisman and Fain 1995). According to this hypothesis the absence of functional CNG channels is equivalent to the permanent closure of channels which occur under continuous bright light conditions. Continuous exposure of experimental animals to light has been shown to result in photoreceptor degeneration (Fain and Lisman 1999; Pierce 2001; Wenzel et al. 2005). It is unclear, how constitutive activation of phototransduction leads to retinal degeneration. Low Ca^{2+} levels resulting from the loss of Ca^{2+} influx through CNG channels as well as toxicity induced by reactive oxygen species may play a role in this process (Fain 2006; Fain and Lisman 1999; Lisman and Fain 1995; Pierce 2001).

6.1.2 CNGB1a

The CNGB1 gene locus consists of at least 33 exons encoding several splice variants (Ardell et al. 2000). Rods express a 240 kD isoform (CNGB1a) containing a long cytosolic N-terminus that is also translated as a separate cytosolic protein (glutamic acid rich protein, GARP) (Chen et al. 1993; Körschen et al. 1995; Sugimoto et al. 1991). Shorter variants of the subunit lacking the GARP-part are present in the native olfactory CNG channel (CNGB1b) (Bönigk et al. 1999; Sautter et al. 1998), and have been identified in sperm cells and other tissues (Biel et al. 1996; Wiesner et al. 1998). A CNGB1-deficient mouse model covering all potential CNGB1 splice variants was generated by deleting exon 26 which encodes the pore-forming region and the S6 segment (Hüttl et al. 2005). These mice show an impairment of both vision and olfaction. In this section we will focus on the retinal phenotype.

CNGB1-deficient mice show a slow-progressing degeneration of the retina caused by apoptotic death and concurred by retinal gliosis. The degeneration primarily affects rod photoreceptors and is already detectable in these cells at postnatal day 15 (P15). Cones are quite unaffected up to an age of six months but start to degenerate subsequently. At the age of about one year, the retina of CNGB1-deficient animals is essentially devoid of both rods and cones. The principal structure of the inner retina is unaffected by the deletion of CNGB1. However, rod bipolar cells and horizontal cells reveal some morphological alterations (e.g. sprouting extensions, retraction of processes and misplacement of cell bodies). CNGB1-deficient mice show no detectable rod-mediated responses in ERGs, whereas up to six months of age cone responses are normal. Later on, the cone responses also decrease and at the age of 12 months knockout mice are virtually blind. In agreement with the ERG measurements, the vast majority of isolated rod photoreceptors of young mice (P17) fail to respond to light. Only in a few cells a tiny dark current can be detected. The severe retinal phenotype of CNGB1-deficient mice was unexpected given that CNGB1 was originally considered to be a modulatory subunit which is not needed for principal channel formation. This conundrum could be solved by Western blots and immunohistochemistry which showed that in CNGB1-deficient mice only trace amounts of the CNGA1 subunit are present in rod outer segment. Thus, in contrast to findings in Xenopus oocytes and in HEK293 cells, CNG channels expressed in native rod photoreceptors cannot be assembled by four CNGA1 subunits likely because targeting of homomeric channels to the rod-outer segment is disrupted. The mechanism of cell death may be analogous to that discussed above for CNGA1-channelopathies. It is not known why photoreceptors unlike Xenopus oocytes or HEK293 cells cannot efficiently target homomeric CNGA1 channels to the plasma membrane. In any case, it is tempting to speculate that the requirement of CNGB1 for targeting CNGA1 to the outer segment may have evolved to ensure that only heteromeric channels reach the outer segment membrane.

The total loss of CNG channel expression explains why the retinal phenotype of CNGB1-deficient mice is so reminiscent of that found in RP patients with null mutations in the CNGA1 gene. Interestingly, RP patients have been recently identified who carry a point mutation in the CNBD of CNGB1 (Bareil et al. 2001). So far,

the functional consequences of this mutation, G993V, which involves a highly conserved residue in the CNG channel family, have not been determined in expression systems. Importantly, it is not known whether the mutant protein is targeted to the plasma membrane of rod outer segments. If the mutant protein is not targeted, the disease mechanism in CNGB1-deficient mice would be equivalent to the one leading to human CNGB1-related RP. Alternatively, if the mutant CNGB1 is produced and assembled with CNGA1, it might impair the cGMP-dependent activation of the channel.

6.2 Cone Photoreceptor CNG Channel

6.2.1 CNGA3

Mutations in the CNGA3 subunit cause achromatopsia (total colorblindness or rod monochromacy) (Kohl et al. 1998). CNGB3 (see below) and GNAT2 (encodes cone-specific transducin alpha subunit) have been identified as additional achromatopsia genes (Aligianis et al. 2002; Kohl et al. 2000, 2002; Sundin et al. 2000). Achromatopsia is a rare autosomal recessive disorder (incidence about 1 in 30,000), in which functional cones are absent from the retina (Sharpe et al. 1999). Affected individuals show a total loss of color discrimination, photophobia, nystagmus and poor visual acuity. Electroretinography reveals absent photopic (cone) responses and normal scotopic (rod) responses. Individuals with incomplete achromatopsia retain some color vision (Pokorny et al. 1982). About 50 mutations have been identified in the CNGA3 gene of patients suffering from achromatopsia (Eksandh et al. 2002; Johnson et al. 2004; Kellner et al. 2004; Michaelides et al. 2004; Nishiguchi et al. 2005; Varsanyi et al. 2005; Wissinger et al. 2001). Most of the mutations are amino acid substitutions compared to a few stop-codon mutations and deletions or insertions. The effect of several mutations on channel function was examined in heterologous expression systems. CNGA3 subunits carrying mutations in the S1 region (Y181C, N182Y, L186F and C191Y) fail to be targeted to the plasma membrane indicating that the folding and/or subunit assembly was disturbed in these mutants (Patel et al. 2005). Recently, a Japanese patient with congenital achromatopsia was found to carry a point mutation within the carboxy-terminal leucine zipper (CLZ) domain (L633P) (Goto-Omoto et al. 2006), which is important for proper heteromeric assembly of CNG channels (Zhong et al. 2003). Profoundly impaired channel targeting may also explain the loss of function induced by several other mutations. Alternatively, mutations may alter the biophysical properties of the channels. For example, the N471S and R563H mutants were found to have an increased cGMP affinity which may prevent complete closure of the channels in response to light stimulation (Liu and Varnum 2005). Consequently, these "gain-of-function" mutations may lead to a sustained and potentially toxic elevation of intracellular Ca^{2+} levels in cone outer segments. Another example of a mutation that

leads to a channel with altered biophysical properties has been recently reported by Tränkner and collaborators (2004). The authors investigated a mutation in the pore region of CNGA3 (T369S) which is linked to incomplete achromatopsia. When co-expressed with the CNGB3 subunit, this mutant induced robust currents. However, the channel revealed a lower efficacy of Ca^{2+} blockage at physiological membrane potentials which tentatively would lead to a lower signal-to-noise ratio of the light response and, hence explain the lower light sensitivity observed in the patients.

CNGA3-deficient mice display the principal hallmarks of achromatopsia described above (Biel et al. 1999). As the only exception, deletion of CNGA3 has no obvious effect on visual acuity (Schmucker et al. 2005). This finding may be explained by the fact that mice lack the cone-enriched macula that confers high-acuity vision in humans (Szel et al. 2000). CNGA3-deficient mice reveal a complete loss of cone function. By contrast, the rod photoreceptor system remains fully intact (structurally and functionally) during the whole life span of the animals. CNGA3-deficient cones show a delayed postnatal migration behavior, form irregular synapses and reveal a progressive degeneration (Claes et al. 2004; Michalakis et al. 2005). Also the postsynaptic partners (e.g. cone bipolar cells) react to the missing cone input and form ectopic synapses with rods (Haverkamp et al. 2006). Interestingly, dorsal cones expressing medium-wave sensitive opsin survive significantly longer (up to 22 months) than ventral cones expressing mostly short-wave sensitive opsin (less than 3 months). The reason for this difference remains to be determined. A comparable asymmetrical pattern of cone loss was observed in the retina of mice lacking the guanylyl cyclase E (GC-E) (Coleman et al. 2004). CNGA3-deficient cones fail to transport opsins into outer segments, downregulate various proteins of the phototransduction cascade and induce apoptotic death (Michalakis et al. 2005). As discussed above for CNGA1/B1-deficient rods, cones of CNGA3-deficent mice may degenerate as a result of the non-functional visual cascade. Alternatively, the CNG channel may serve as an important structural protein in outer segments. Although proteins binding to the cone channel have not yet been identified it is reasonable to assume that the cone channel like the related rod CNG channel (Kang et al. 2003; Poetsch et al. 2001; Schwarzer et al. 2000) is part of a highly structured protein complex. Thus, the loss of CNGA3 would lead to a structural alteration of cone outer segments. The accumulation of opsins in the inner segments and somata may result from the failure of these proteins to be targeted into these irregular outer segments. Given the high expression levels of opsins the mislocalization and accumulation of these proteins could well induce cellular stress and apoptosis. Misrouting and accumulation of proteins has been found frequently in degenerative processes in neurons (Taylor et al. 2002).

The CNGA3 subunit has been identified in a number of tissues different from retina, including a subpopulation of ORNs in the olfactory epithelium, sperm, kidney and brain (for overview (Kaupp and Seifert 2002)). CNGA3-deficient mice reveal normal fertility indicating that CNGA3 is not essential for sperm function (Biel et al. 1999).

6.2.2 CNGB3

A missense mutation in the CNGB3 gene (S435F) was identified in color-blind individuals originating from the Pingelap Atoll of Micronesia (Sundin et al. 2000). In this small island, achromatopsia is very frequent and affects nearly 10% of the native population (Sacks 1997). Additional mutations of the CNGB3 gene have been identified in other patients with achromatopsia, most of which lead to truncated proteins (Bright et al. 2005; Goto-Omoto et al. 2006; Johnson et al. 2004; Kohl et al. 2000, 2005; Michaelides et al. 2004; Sundin et al. 2000; Varsanyi et al. 2005). Overall, mutations in the CNGB3 gene account for about 50% of all cases of achromatopsia (Kohl et al. 2005). A missense mutation in exon 6 of the CNGB3 gene (D262N corresponding to human D267N)) and a CNGB3 null-deletion were also identified in two canine inbred strains with cone degeneration (Sidjanin et al. 2002).

Several disease-associated mutations have been functionally characterized in Xenopus oocytes (Bright et al. 2005; Peng et al. 2003) or HEK293 cells (Okada et al. 2004). Interestingly, some mutations resulted in an increase in apparent affinity for cAMP/cGMP, e.g. the S435F mutation. CNGA3/CNGB3$_{S435F}$ heteromeric channels exhibited a fourfold increase in cAMP affinity and a twofold increase in apparent affinity for cGMP. In addition, the mutant channel had a slightly higher open probability in the presence of saturating cyclic nucleotide concentration than wild-type channels. It is unclear whether these subtle effects can account for the severe clinical phenotype of the patients. Alternatively, it is possible that the mutation prevents correct protein folding and/or assembly with the CNGA3 subunit in vivo. The finding that null mutations of the CNGB3 gene induce achromatopsia strongly suggests that the CNGA3 subunit (like the CNGA1 subunit) is not able to form homomeric channels in vivo.

6.3 Olfactory CNG Channel

6.3.1 CNGA2

Unlike all other CNG channel genes, the CNGA2 gene is localized on the X chromosome. So far mutations of this gene have not been identified in humans. However, four different CNGA2-deficient mouse lines have been generated to define the physiological role of this subunit (Baker et al. 1999; Brunet et al. 1996; Zhao and Reed 2001; Zheng et al. 2000). CNGA2-deficient mice display elevated neonatal lethality due to their inefficiency to find their mother's nipples to suckle milk. Indeed, initial electroolfactogram (EOG) recordings showed that the mice exhibit no detectable responses to odorants (Brunet et al. 1996). Moreover, in perforated patch experiments ORNs of CNGA2-deficient mice did not respond to odors or activators of the cAMP system (IBMX/forskolin) (Delay and Restrepo 2004). Together, these findings indicate that olfaction is conferred by a cAMP/CNGA2-mediated signaling pathway.

In addition, the results strongly argue against a major contribution of other signaling cascades which have been suggested in the past (e.g. IP3-mediated olfaction; (Boekhoff et al. 1990)). More recent studies confirmed this principal conclusion but also uncovered that CNGA2-deficient mice are not totally anosmic. Zhao and Reed (2001) found very small but reproducible EOG responses to a variety of odorants in CNGA2-deficient mice. In behavioral tests CNGA2-deficient mice could detect and discriminate several odorants, including 2-heptanone and 2,5-dimethylpyrazine (Lin et al. 2004). The molecular components conferring this residual olfaction remains to be determined. Pharmacological blockers suggest that the respective signaling pathway(s) are cAMP-independent. GC-D type ORNs (see Chap. 3) could contribute to the residual odorant responses observed in CNGA2-deficient mice.

Beside its key role in olfactory transduction, CNGA2 is required for normal development of the olfactory epithelium and olfactory bulb (Baker et al. 1999). In comparison to wild-type mice, CNGA2-knockouts display a thinner olfactory epithelium and show lower expression of an olfactory marker protein (OMP). Moreover, the olfactory bulb is smaller in size, and tyrosine hydroxylase expression is reduced in most periglomerular neurons of the main olfactory bulb, but is retained in necklace-glomeruli.

In the olfactory system, sensory neurons expressing a given odorant receptor (OR) project with precision to a pair of spatially invariant glomeruli within the olfactory bulb (Ressler et al. 1994; Vassar et al. 1994). Gene targeting experiments indicate that odorant receptors are required for precise targeting (Wang et al. 1998). In contrast, the olfactory CNG channel and hence, odorant-induced electrical activity, does not seem to be required for proper axon guidance (Lin et al. 2000; Zheng et al. 2000). However, this paradigm may not hold true for all subtypes of ORNs. It was reported that CNGA2-deficient ORNs expressing the M72 OR projected to 4–6 instead of 2 glomeruli in the wild-type mouse suggesting that at least in M72 OR neurons CNGA2 may be important for correct axon targeting (Zheng et al. 2000). In addition, CNGA2$^{-/-}$ mice fail to mate or fight, suggesting a broad and essential role of CNGA2-mediated olfaction in regulating these behaviors (Mandiyan et al. 2005).

CNGA2 is expressed only in the main olfactory epithelium (MOE) but not in the vomeronasal organ this suggests a broad and essential role for the MOE in regulating these behaviors.

The olfactory CNG channel was identified in murine hippocampus suggesting a role of this protein in memory formation (Bradley et al. 1997; Kingston et al. 1996; Leinders-Zufall et al. 1995). In support of this hypothesis, hippocampal long-term potentiation (LTP) evoked by a theta burst stimulation protocol was reduced in CNGA2-deficient mice (Parent et al. 1998). By contrast, several measures of basal synaptic activity as well as LTP induced by high-frequency stimulation seemed to be similar in wild-type and knockout mice. Behavioral tests to determine the memory performance of CNGA2-deficient mice have not been reported so far leaving open whether or not the observed alterations of hippocampal LTP are physiologically relevant.

6.3.2 CNGA4

Mice deficient for the CNGA4 subunit are fertile and show no obvious morphological differences with respect to wild-type mice (Kelliher et al. 2003; Munger et al. 2001). Moreover, the olfactory epithelium and the olfactory bulb develop normally in these mice. Maximal EOG responses induced by IBMX, an inhibitor of the phosphodiesterase, are comparable in both genotypes. However, the dose response curves are shifted to about threefold higher IBMX concentrations in the CNGA4-deficient mice suggesting that the mutant CNG channel is less sensitive to cAMP. Indeed, the apparent cAMP affinity of the CNG channel as determined from excised patch recordings is about tenfold lower in the knockouts than in wild-type mice. Thus, CNGA4 is clearly required to confer high cAMP affinity to the olfactory channel. CNGA4-deficient mice also show a defect in odor adaptation (Kelliher et al. 2003; Munger et al. 2001). The decay time of the EOG in response to an odorant stimulus is profoundly slower in knockouts than in wild-type mice. Moreover, in paired-pulse stimulations both EOG responses are identical in the knockout mice, whereas the second EOG is always smaller (due to adaptation) in the wild-type animals. Electrophysiological recordings in excised patches from ORNs clarified why CNGA4 is needed for odor adaptation. The recordings revealed that in the absence of CNGA4 the kinetics of CaM-dependent inhibition is profoundly slower than in its presence. Thus, CNGA4 is required for fast feedback-inhibition of channel activity by CaM. The result also suggested the CaM binding site identified in the CNGA2 subunit (Liu et al. 1994) is probably not involved in CaM-mediated inhibition in vivo.

6.3.3 CNGB1b

RP patients carrying the G993V mutation in the CNGB1 gene were reported to show no olfactory impairment (Bareil et al. 2001). In contrast, CNGB1-deficient mice that lack both the retinal CNGB1a and the olfactory CNGB1b subunit reveal a profound olfactory phenotype (Michalakis et al. 2006). As reported for CNGA2-deficient mice, CNGB1-knockouts show an increased postnatal mortality and delayed body weight gain that is most probably due to a deficit in locating their mother's nipples to suckle milk. The knockout mice have a decreased olfactory performance and their EOG responses display a reduced maximal amplitude and decelerated onset and recovery kinetics compared to wild-type mice (Michalakis et al. 2006). Moreover, fast odor adaptation is impaired confirming that CNGB1 is absolutely necessary for CaM-dependent inhibition. Interestingly, the levels of CNGA2 and CNGA4 protein are dramatically reduced in the cilia of CNGB1-deficient mice. In contrast, other ciliary proteins (e.g. adenylyl cyclase III) are normally expressed. Further analysis of these mice showed that CNGA2/CNGA4 containing channels are assembled and also inserted into the plasma membrane of the knobs but do not localize to the cilia. Experiments with MG-132, a specific proteasome inhibitor, suggest that these channels are degraded by the action of the proteasome (Michalakis et al. 2006). A recent

study analyzed the ciliary targeting of CNG channels in Madin-Darby canine kidney (MDCK) epithelial cells (Jenkins et al. 2006). By overexpressing wild-type and mutated olfactory CNG channel subunits in these cells a RVXP-motif in the C-terminus of CNGB1b was identified that is necessary but not sufficient for ciliary targeting of CNGA2 (Jenkins et al. 2006). Interestingly, the same report found that CNGA4 was not needed for ciliary CNG channel targeting in MDCK cells. In contrast, analysis of CNGA4$^{-/-}$ mice revealed a ciliary targeting defect similar to that present in CNGB1-knockout mice (Michalakis et al. 2006). Thus, unlike MDCK cells native ORNs require both CNGA4 and CNGB1b for normal ciliary targeting of CNGA2.

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cGMP Regulated Protein Kinases (cGK)

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Abstract cGMP-dependent protein kinases (cGK) are serine/threonine kinases that are widely distributed in eukaryotes. Two genes – *prkg1* and *prkg2* – code for cGKs, namely cGKI and cGKII. In mammals, two isozymes, cGKIα and cGKIβ, are generated from the *prkg1* gene. The cGKI isozymes are prominent in all types of smooth muscle, platelets, and specific neuronal areas such as cerebellar Purkinje cells, hippocampal neurons, and the lateral amygdala. The cGKII prevails in the secretory epithelium of the small intestine, the juxta-glomerular cells, the adrenal cortex, the chondrocytes, and in the nucleus suprachiasmaticus. Both cGKs are major down stream effectors of many, but not all signalling events of the NO/cGMP and the ANP/cGMP pathways. cGKI relaxes smooth muscle tone and prevents platelet aggregation, whereas cGKII inhibits renin secretion, chloride/water secretion in the small intestine, the resetting of the clock during early night, and endochondreal bone growth. cGKs are also modulators of cell growth and many other functions.

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Abbreviations

cGMP-dependent protein kinases, cGK; amino acids, aa; guanylyl cyclase, sGC; soluble guanylyl cyclase, sGC; particulate guanylyl cyclase, pGC; atrio-natriuretic peptide, ANP; brain-natriuretic peptide, BNP; C-type natriuretic peptide, CNP; phosphodiesterases, PDE; cyclic nucleotide-gated, CNG; inositol 1,4,5-trisphosphate receptor I (IP_3RI)-associated cGMP kinase substrate, IRAG; regulator of G protein 2, RGS2; cystic fibrosis transmembrane conductance regulator, CFTR; sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, SERCA; Ca^{2+}-activated maxi-K^+ channel, BK_{Ca}; regulatory myosin phosphatase targeting subunit, MYPT; myosin light chain phosphatase, MLCP; smooth muscle, SM; SM-myosin heavy chain, SM-MHC; SM-α-actin, SMA; nucleus suprachiasmaticus, SCN; circadian time, CT; Na^+/H^+ exchanger 3, NHE3.

1 Introduction

NO is generated by three different isozymes (NO-synthases; NOS 1–3). In many cells, NO increases the concentration of cyclic guanosine monophosphate (cGMP) by activation of the soluble guanylyl cyclase (sGC) (Friebe and Koesling 2003; Katsuki et al. 1977). cGMP is also generated by membrane-bound particulate guanylyl cyclases (pGCs, e.g. GC-A, GC-B, and GC-C). GC-A and GC-B are the major receptors for a family of natriuretic peptides released from the heart and vascular endothelium, like atrio-natriuretic peptide (ANP), brain-natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), whereas GC-C is the receptor for guanylin, an intestinal peptide involved in intestinal fluid regulation (Garbers 1992). Further analysis of the cGMP system identified a number of intracellular targets for cGMP. For example, cGMP binds to cyclic adenosine monophosphate (cAMP)-specific phosphodiesterases (PDEs) and thereby modulates the concentration of cAMP enabling a cross-talk between both cyclic nucleotide pathways (Bender and Beavo 2006; Rybalkin et al. 2003). cGMP and cAMP activate cyclic nucleotide-gated (CNG) cation channels that are an important part of the signal transduction pathway in the visual and olfactory system (Biel et al. 1999; Hofmann et al. 2004). Most cells contain at least one of three cGMP-dependent protein kinases (cGKs): cGKIα, cGKIβ, or cGKII (Feil et al. 2003; Hofmann 2005; Pfeifer et al. 1999) that are targeted by their distinct amino termini to different substrates that are involved in the regulation of different cellular functions.

NO signals not only through the cGMP pathway but has several effects that are independent of the cGMP/cGK signalling pathway (Hess et al. 2005). Interpretation of cGMP effects should include the same precautions, because (1) cGMP has several effectors that may be used simultaneous in various tissues, (2) cGMP might activate directly or indirectly cAMP-dependent protein kinases, and (3) some of the effects of "cGK-specific" activators and inhibitors are not mediated by cGKs (Burkhardt et al. 2000; Daugirdas et al. 1991; Marshall et al. 2004; Wyatt et al. 1991b).

This article will concentrate on some results obtained by total or tissue-specific deletion of the cGK genes and of some of their substrates. We will discuss how cGK signalling might contribute to the maintenance of a "healthy" and physiological status on the one hand and at the same time might affect many aspects that modulate the progression of various diseases. Please note that the physiological function of cGKs are also summarized in the chapters on "cGK Substrates" by J. Schlossmann, "cGMP Kinase-Modulators" by E. Butt, "cGMP kinase as behaviour modifier" by M Sokolowski, "Platelets and blood cells" by U Walter, and "cGMP Signalling in Brain" by R Feil. In addition, several excellent reviews are available for those wanting to delve deep into this topic (Feil and Kleppisch 2008; Feil et al. 2003; Hofmann 2005; Hofmann et al. 2006; Lincoln et al. 2006; Lohmann and Walter 2005; Pfeifer et al. 1999).

2 Properties of cGKs

2.1 Genes, Isozymes, and Structure

cGKs belong to the family of serine/threonine kinases and are present in a variety of eukaryotes ranging from the unicellular organism *Paramecium* to *Homo sapiens* (Francis and Corbin 1999; Pfeifer et al. 1999). Mammals have two cGK genes, *prkg1* and *prkg2* that encode cGKI and cGKII (Orstavik et al. 1997; Sandberg et al. 1989; Wernet et al. 1989). The human *prkg1* gene is located on chromosome 10 at p11.2–q11.2 (52,421,124 bp–53,728,116 bp) and has 15 exons. The N-terminus (the first 90–100 aa) of cGKI is encoded by two alternative exons that produce the isoforms cGKIα and cGKIβ. Their transcripts code for 671 aa (cGKIα) and 686 aa (cGKIβ) yielding proteins with an apparent mass of 76,364 Da (cGKIα) and 77,804 Da (cGKIβ), respectively. The human *prkg2* gene is located on chromosome 4 at q13.1–q 21.1 (82,228,861 bp–82,355,212 bp) and has 19 exons. Its transcript length is 3,431 bp (number of residues 762) yielding a protein with an apparent mass of 87,432 Da.

The enzymes have a rod like structure. They are composed of two functional domains: a regulatory (R) domain and a catalytic (C) domain (Fig. 1). The regulatory domain is further subdivided into the N-terminal domain and the cGMP binding domain containing the high (cGMP I) and low (cGMP II) affinity binding pockets. The two tandem cGMP-binding sites interact allosterically (for details see Francis and Corbin 1999; Pfeifer et al. 1999). Occupation of both binding sites induces a large change in secondary structure (Landgraf et al. 1990) to yield a more elongated molecule (Wall et al. 2003; Zhao et al. 1997). The catalytic domain contains the MgATP- and peptide-binding pockets. Binding of cGMP to both sites in the R-domain releases the inhibition of the catalytic center by the N-terminal autoinhibitory/pseudosubstrate domain and allows the phosphorylation of serine/threonine residues in target proteins. Activation of substrate phosphorylation may be preceded

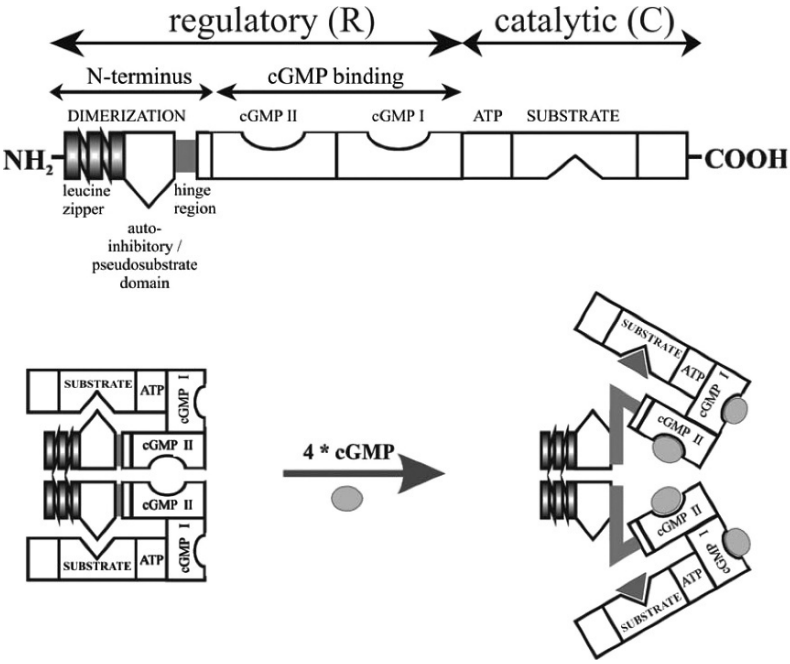

Fig. 1 Structure of cGMP-dependent protein kinase. For details see text

by autophosphorylation of the amino-terminal autophosphorylation site of cGK. Autophosphorylation increases the spontaneous activity of cGKI and cGKII (Francis et al. 2002; Smith et al. 1996; Vaandrager et al. 2003; Wyatt et al. 1991a) and is initiated by the binding of low cGMP concentrations to the high affinity site of cGKI (Hofmann et al. 1985; Smith et al. 2000). In addition to controlling activation and inhibition of the catalytic center, the N-terminus has two other functions: (1) dimerization – cGKs are homodimers that are held together by a leucine zipper present in the N-terminus; (2) targeting – the enzymes are targeted to different subcellular localizations by their N-termini.

The two cGKI isozymes are activated at submicromolar to micromolar concentrations of cGMP with cGKIα being significantly more sensitive than the cGKIβ isoform (Gamm et al. 1995; Ruth et al. 1997). The different activation constants are caused by differences in the leucine zipper, the autoinhibitory/pseudosubstrate domain, and the hinge region. The two cGKI isozymes share 8 cysteines outside of the amino terminus. The cGKIα contains additional cysteines at aa 43 and aa 118. Oxidation of the bovine cGKIα enzyme by Cu^{2+} activates the enzyme through formation of two disulfide bonds between Cys-118 and Cys-196 and Cys-313 and Cys-519 (Landgraf et al. 1991). The biological significance of this activation is not obvious, because oxidation of the enzyme by transition metals is unlikely to occur in vivo. Surprisingly, the cGKIα forms an interprotein disulfide at Cys-43 linking its two subunits in cells exposed to exogenous hydrogen peroxide (Burgoyne et al.

2007). Cys 43 is only present in the Iα isoform. Oxidation of Cys 43 activates the kinase independent of cGMP and has been suggested as an alternative activation mechanism of cGKI in vascular smooth muscle under oxidative stress.

2.2 Tissue Distribution

cGKI is present in high concentrations ($>0.1\,\mu M$) in all smooth muscles, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular endplate, and kidney. Low levels have been identified in cardiac muscle, vascular endothelium, granulocytes, chondrocytes, osteoclasts, and a diverse number of brain nuclei (Feil et al. 2005; Keilbach et al. 1992; Lohmann et al. 1981). The Iα isozyme is found in lung, heart, dorsal root ganglia, and cerebellum. Together with the Iα isozyme, the Iβ isozyme is highly expressed in smooth muscle, including uterus, vessels, intestine, and trachea (Geiselhöringer et al. 2004a; Weber et al. 2007). Platelets, hippocampal neurons, and olfactory bulb neurons contain mainly the Iβ isozyme (Geiselhöringer et al. 2004a). The Iα and Iβ cGKs are soluble enzymes and interact with different proteins through their distinct N-termini. In contrast, cGKII is anchored at the plasma membrane by myristoylation of the N-terminal Gly2 residue. Only the membrane-bound cGKII phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) thereby activating intestinal chloride secretion (Vaandrager et al. 1998). In addition, cGKII is expressed in several brain nuclei, intestinal mucosa, kidney, adrenal cortex, chondrocytes, and lung (de Vente et al. 2001; el-Husseini et al. 1995; Lohmann et al. 1997; Werner et al. 2004).

2.3 Substrates and Signalling Mechanisms

Over ten substrates have been identified that are phosphorylated in vivo by cGKI and several that are modified by cGKII (See Table 1 for details). In addition, a number of proteins exist that are proven substrates in expression system (Table 2). So far, their phosphorylation has not been shown to occur in vivo. The majority of identified substrates modulate the function of other signalling pathways being ion channels, G proteins, and associated regulators or cytoskeletal associated proteins. Details on the function of these proteins will be given in the following sections and in the chapter on "cGK Substrates" and further chapters. The substrate specificity depends on the distinct N-terminus of each isozyme, e.g. the inositol 1,4,5-trisphosphate receptor I (IP_3RI)-associated cGMP kinase substrate (IRAG) interacts only with the N-terminus of the Iβ isozyme (Schlossmann et al. 2000), whereas the myosin phosphatase binding subunit interacts specifically with the N-terminus of the Iα isozyme (Surks et al. 1999). Inhibition of IP_3 synthesis depended specifically on the expression of the Iα isozyme (Ruth et al. 1993; Tang et al. 2003) and could not be achieved by expression of the Iβ isozyme (Meinecke et al. 1994) presumably because only

Table 1 Established cGK substrates and their physiological function(s)

Substrate	Molecular weight (kDa)	cGK iso-form	Tissue or cells	Function of phosphorylation	Reference
BK_{Ca}	130	cGKI	SM	Increased open probability; membrane hyperpolarization	(Sausbier et al. 2000)
G-substrate	32	cGKI	Cerebellum	Protein phosphatase inhibitor; initiation of LTD	(Endo et al. 2003)
IP_3 receptor type I	230	cGKI	Cerebellum	Stimulation of calcium release from IP_3 sensitive stores	(Haug et al. 1999; Wagner et al. 2003)
IRAG	125	cGKIβ	SM	Reduced calcium release from IP_3 sensitive stores	(Geiselhöringer et al. 2004b; Schlossmann et al. 2000)
MYPT1	130	cGKIα	SM	Inhibition of myosin phosphatase inhibition by rho kinase; decreased calcium sensitization	(Wooldridge et al. 2004)
PDE5	100	cGKI	SM, Pl	Enhanced cGMP degradation	(Rybalkin et al. 2002)
Phospho-lamban	6	cGKI	Vascular SM	Enhanced calcium uptake by the Ca-ATPase Serca, faster relaxation?	(Lalli et al. 1999)
RGS2	24	cGKIα	SM	Inhibition of IP_3 generation	(Sun et al. 2005; Tang et al. 2003)
RhoA	22	cGKI	SM, Hippo	Reduced MLC phosphorylation, vesicle trafficking	(Ellerbroek et al. 2003)
Sox9	56	cGKII	Chondrocytes	Bone development	(Chikuda et al. 2004)
Telokin	17	cGKI	SM	Inhibition of MLCK activity	(Walker et al. 2001)
VASP	46/50	cGKI	SM, Pl, Hippo	Regulation of the actin cytoskelet, vesicle trafficking	(Butt et al. 1994; Hauser et al. 1999)
Vimentin	57	cGKI	Neutrophils	Regulation of the cytoskeleton	(Pryzwansky et al. 1995)

Abbreviation: SM, smooth muscle; Pl, platelets; Hippo, hippocampus

the Iα isozyme binds to the regulator of G protein 2 (RGS2) (Tang et al. 2003) and terminates thereby the activity of phospholipase C. The above mentioned interaction of CFTR is specific for the N-terminus of cGKII (Vaandrager et al. 1998). Recent evidence suggested that Ser-293 of VASP, the cGKI-specific site, is also phosphorylated by cGKII in primary renal tubule epithelial cells (Lindsay et al. 2007) indicating that the cGKs might have an overlapping substrate specificity. The cGKI

Table 2 cGK substrates phosphorylated in heterologous systems and with a potential function in vivo

Substrate	Molecular weight (kDa)	cGK Iso-form	Tissue or cells	Potential function of phosphorylation	Reference
Cav1.2 β2a subunit S496	74	cGKIα	HEK cells	Inhibition of L-type calcium current	(Yang et al. 2007)
CFTR	200	cGKII	IEC-CF7cells (Intestinal cell line)	Stimulation of chloride channel	(Vaandrager et al. 1998)
CRP2/CRP4	23	cGKIβ	SM, Neurons	Regulation of smooth muscle tone and smooth muscle specific gene expression; pain perception	(Huber et al. 2000; Schmidtko et al. 2008; Zhang et al. 2007)
Hsp27	27	cGKI	Pl	Decrease of actin polymerisation in vitro	(Butt et al. 2001)
Septin-3	40	cGKI	Brain	Vesicle trafficking	(Xue et al. 2004)
Rap1GAP2	90	cGKI	Pl	Inhibition of Rap1	(Schultess et al. 2005)
Serotonin transporter T276	70	cGK	Brain, CHO-1 cells	Increased serotonin uptake	(Ramamoorthy et al. 2007)
TRPC3	97	cGKI	HEK293	Inhibition of store operated calcium influx	(Kwan et al. 2004)
TxA2 receptor Iα	40	cGKI	HEK293	Desensitization of TP Iα signaling	(Reid and Kinsella 2003)
VASP S239	46/50	cGKII	Primary renal tubule epithelial cells	Loss of lamellipodial protrusion and cell rounding	(Lindsay et al. 2007)
GSK-3β S9	47	cGKII	Chondrocyte/ osteosarcoma	Correct chondrocytes hypertrophy in skeletal bone growth/increase in C/EBPβ binding	(? ?; Zhao et al. 2005)

Abbreviation: SM, smooth muscle; Pl, platelets

isozyme specificity has been tested recently in two transgenic mouse lines expressing only either the cGKIα or the cGKIβ isoform in all smooth muscles (Weber et al. 2007). Both mice lines reconstituted the basic functions of cGKI in several test systems raising the possibility that the substrate specificity is less stringent in vivo than in vitro.

3 cGK Signalling in the Cardiovascular System

3.1 Cardiac Contractility and Remodeling

It is well established that NO and natriuretic peptides (NPs) play an important role in cardiovascular health and disease (D'Souza et al. 2004; Garbers and Dubois 1999; Hofmann et al. 2004; Ignarro 2002; Lloyd-Jones and Bloch 1996; Massion et al. 2003). NO and ANP relax small arteries and arterioles resulting in decreased blood pressure, and NO prevents acute vasoconstriction and thrombosis. In addition, NO/NP signalling modulates cardiac and vascular remodelling processes that are associated with congestive heart failure and atherosclerosis. The molecular mechanisms of cardiovascular NO/NP signalling are not well understood, although cGMP-elevating drugs such as glyceryl trinitrate have been used successfully for the treatment of angina pectoris (Parker and Parker 1998) and the PDE5 inhibitor sildenafil for the treatment of erectile dysfunction and pulmonary hypertension (Lincoln 2004). It is currently accepted that many effects of NO/NP are mediated, at least in part, via cGMP-dependent pathways (Kuhn 2003; Mullershausen et al. 2003; Rybalkin et al. 2003). The major cGK expressed in the cardiovascular system is cGKI (Feil et al. 2003).

The relative importance of cGMP-dependent and cGMP-independent NO effects is controversial (Hare and Stamler 2005; Massion et al. 2003). The combined analysis of conventional and cardiomyocyte-specific cGKI knockout mice demonstrated that cGMP/cGKI contributes to the negative inotropic effect of NO in the juvenile as well as in the adult murine heart (Wegener et al. 2002). However, the NO/cGMP/cGKI pathway does not appear to be involved in the negative inotropic action of acetylcholine (Godecke et al. 2001; Vandecasteele et al. 1999; Wegener et al. 2002). Cardiomyocyte-directed overexpression of cGKIα augmented NO/cGMP inhibition but not muscarinic inhibition of L-type Ca^{2+} channel activity (Schroder et al. 2003). These findings are in line with the results obtained with cGKI-deficient mice and suggest a mechanism for the negative inotropic action of cGKI, namely the inhibition of L-type Ca^{2+} channels. Interestingly, CNP can exert a positive inotropic effect, and this effect was enhanced in the cGKIα overexpressing mice (Wollert et al. 2003). Thus, it appears that NO-stimulated cGMP inhibits, whereas CNP-stimulated cGMP increases, cardiac contractility, and both effects are mediated via cGKI. The dual effects of cGKI on cardiac contractility might be related to distinct cells (e.g. cardiomyocytes versus fibroblsasts), or sub cellular localization of the NO/cGMP/cGKI *versus* CNP/cGMP/cGKI signalling and differences in the amount and duration of cGMP synthesized. We can also not exclude the possibility that yet unidentified alternative signals mediate in concert with cGMP/cGKI the biological effects by either NO or CNP.

The development of cardiac hypertrophy and congestive heart failure is associated with an altered sarcomere organization, fibrosis, and the expression of several fetal genes such as ANP and BNP (Stein and Levin 1998). Mice lacking ANP or the ANP receptor, GC-A, develop pressure-independent cardiac hypertrophy (Holtwick

et al. 2003; John et al. 1995; Kishimoto et al. 2001; Knowles et al. 2001; Oliver et al. 1997). The hypertrophic response of cultured neonatal rat ventricular myocytes to α_1-adrenergic stimulation is suppressed by ANP, NO, or cGMP (Calderone et al. 1998). These results indicate that stimulation of cGMP synthesis by ANP or NO inhibits cardiomyocyte hypertrophy. Whether or not the anti-hypertrophic effect of cGMP is mediated by endogenous cGKI is presently unclear. Adenoviral overexpression of cGKI inhibits myocyte hypertrophy in vitro (Wollert et al. 2002), at least in part via inhibition of the calcineurin-NFAT pathway (Fiedler et al. 2002). However, neither global nor cardiomyocyte-specific ablation of cGKI affected the development of cardiac hypertrophy under basal conditions or in response to pressure overload (Feil et al. 2003). Recently, it was shown that administration of sildenafil suppresses the development of cardiac hypertrophy in response to pressure overload and can even reverse pre-established cardiac enlargement in the mouse (Takimoto et al. 2005). Surprisingly, the potent anti-hypertrophic effect of sildenafil was linked to an apparent decrease in the myocardial cGMP level and an increase in cGKI activity. Thus, the causal relationship between cGMP, cGKI and the anti-hypertrophic action of sildenafil is not clear. It will be interesting to study the effect of sildenafil on pressure-induced hypertrophy in cardiomyocyte-specific cGKI knockout mice.

3.2 Vasorelaxation and Blood Pressure

Mice deficient for cGKI show impaired NO/cGMP-dependent dilations of large and small arteries indicating that the vasorelaxant effects of NO, NPs and other cGMP-elevating agents are mediated, at least in part, via activation of cGKI (Koeppen et al. 2004; Pfeifer et al. 1998; Sausbier et al. 2000; Weber et al. 2007). cGKI inhibits both hormone receptor-triggered (Pfeifer et al. 1998) and depolarization-induced contraction (Geiselhöringer et al. 2004b) by interfering with phospholipase C activation (Tang et al. 2003), by lowering $[Ca^{2+}]_i$ (Geiselhöringer et al. 2004b; Pfeifer et al. 1998), by decreasing Ca^{2+} sensitization of contraction (Bonnevier et al. 2004; Sauzeau et al. 2000; Somlyo and Somlyo 2003), by promoting a reduction of L-type Ca^{2+} current (Weber et al. 2007) and by unknown mechanisms. VSMCs express both cGKIα and cGKIβ (Feil et al. 2002; Geiselhöringer et al. 2004a; Weber et al. 2007; Wolfe et al. 1989). One established in vivo target for cGKIβ is IRAG, which has been identified in a complex with the smooth muscle IP_3 receptor type 1 and cGKIβ (Schlossmann et al. 2000). Phosphorylation of IRAG by cGKIβ inhibits IP_3-induced Ca^{2+} release from intracellular stores in transfected COS cells and in smooth muscle cells (Ammendola et al. 2001; Geiselhöringer et al. 2004b; Schlossmann et al. 2000). Recently, mice have been generated expressing a mutated IRAG protein that does not interact with the IP_3 receptor (Geiselhöringer et al. 2004b). In aortic smooth muscle cells of 9 week-old and older IRAG mutants, cGMP was unable to suppress hormone-induced increases in $[Ca^{2+}]_i$ and contractility indicating that the cGKIβ/IRAG/IP_3 receptor pathway inhibits hormone receptor-triggered intracellular Ca^{2+} release and contraction in vivo (Fig. 2). However, this

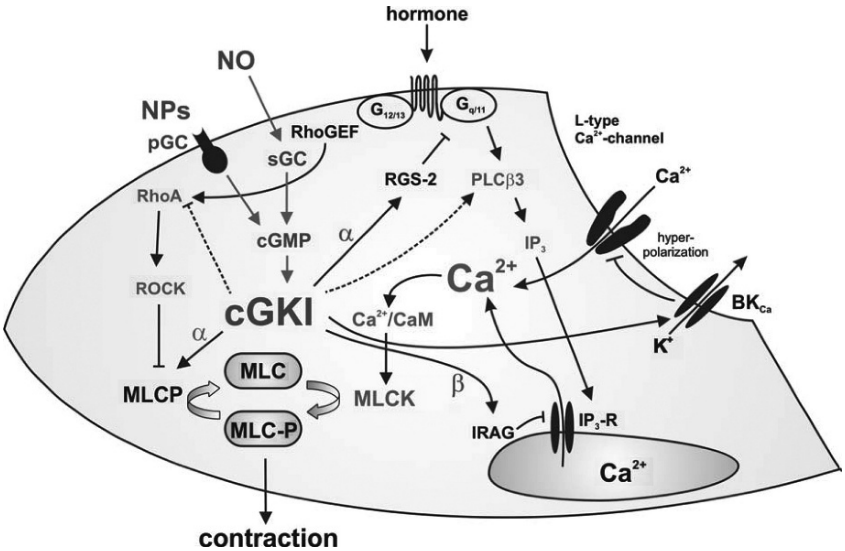

Fig. 2 cGKI Signalling in VSMCs. cGKI-dependent relaxation mechanisms of vascular smooth muscle. Dashed lines, pathways that have not been proven in intact animal tissues. Uninterrupted lines, mechanisms verified in intact animal tissue. Abbreviations are given in the text

pathway is not involved in cGKI-mediated inhibition of contraction initiated by depolarization and activation of voltage-dependent Ca^{2+} channels (Geiselhöringer et al. 2004b).

Additional important cGKI targets that contribute to vasorelaxation (Table 1) have been identified. cGKI activates large-conductance Ca^{2+}-activated maxi-K^+ (BK_{Ca}) channels (Robertson et al. 1993; Sausbier et al. 2000), either by direct phosphorylation (Alioua et al. 1998; Fukao et al. 1999) or indirectly via regulation of a protein phosphatase (White et al. 1993; Zhou et al. 1996). Opening of BK_{Ca} channels results in hyperpolarization of the membrane and closing of voltage-dependent Ca^{2+} channels, thereby, reducing Ca^{2+} influx (Fig. 2). The cGKI may also activate the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), by phosphorylation of the SERCA regulator phospholamban (Koller et al. 2003; Lalli et al. 1999; Raeymaekers et al. 1988). Increased SERCA activity promotes Ca^{2+} re-uptake into the sarcoplasmic/endoplasmic reticulum. The importance of this regulation of the vascular tone is questionable, because deletion of the BK_{Ca} gene (Sausbier et al. 2005) and the phospholamban gene (Lalli et al. 1999) affects the blood pressure only marginally. It has also been suggested that cGKI attenuates early events in hormone receptor-activated pathways including activation of phospholipase C and generation of IP_3 (Hirata et al. 1990; Ruth et al. 1993), perhaps by phosphorylation of receptors (Pfeifer et al. 1998), RGS proteins (Pedram et al. 2000), or phospholipase Cβ (Xia et al. 2001). Reconstitution experiments with cGKI-deficient mice indicated that the inhibition of hormone receptor stimulated Ca^{2+} release can be mediated by the cGKIα and cGKIβ isoforms (Weber et al. 2007). Recently, it has

been shown that the cGKIα isoform binds, phosphorylates, and activates RGS2, which terminates signalling by Gq-coupled receptors for contractile agonists (Tang et al. 2003). These results suggest that, in addition to the cGKIβ/IRAG pathway, the cGKIα/RGS2 pathway may inhibit hormone receptor-triggered Ca^{2+} release and vasoconstriction in vivo (Sun et al. 2005). Another target for cGKI-mediated vasorelaxation is the MLCP (Lee et al. 1997; Surks et al. 1999). The cGKIα isoform interacts with the regulatory myosin phosphatase targeting subunit (MYPT) and activates MLCP (Surks et al. 1999; Wooldridge et al. 2004). Increased MLCP activity would reduce the level of MLC phosphorylation and cause relaxation at constant $[Ca^{2+}]_i$, i.e. Ca^{2+} desensitization of contraction (Fig. 2). Alternative targets of cGKI have been reported (Bonnevier and Arner 2004; Sauzeau et al. 2000; Walker et al. 2001). However, the physiology of the phosphorylation of these proteins remains to be established.

The presented results show that cGKI inhibits receptor-induced vascular smooth muscle contraction by multiple mechanisms including the cGKIβ/IRAG, the cGKIα/RGS2 and the cGKIα/MLCP signalling pathway. The mechanism by which cGKI interferes with depolarization-induced contraction remains controversial. cGKI reduced cardiac Ca^{2+} influx (Mery et al. 1991), most likely by phosphorylation of the calcium channel subunit β2a (Yang et al. 2007) and may contribute thereby to vascular relaxation, because Ca^{2+} influx via the L-type $Ca_v1.2$ calcium channel is essential for sustained contraction of various smooth muscles (Essin et al. 2007; Moosmang et al. 2003; Wegener et al. 2004). The individual contribution of each pathway to cGKI-mediated relaxation presumably varies with the type, the physiological function and the pathology of the vessel.

The effect of cGMP/cGKI signalling on blood pressure is quite complex. Global cGKI-deficiency results in a slight hypertension in young animals, whereas in adult mice the basal blood pressure does not differ between wild type and cGKI-knockout animals (Pfeifer et al. 1998). Furthermore, the smooth muscle specific deletion of the ANP receptor GC-A suggests, that the vascular GC-A is dispensable in the chronic but crucial in the acute regulation of blood pressure by ANP (Kuhn 2005). Interestingly, deletion of eNOS in mice leads to a hypertension in these animals (Huang et al. 1995). Taken together, these studies in SMC GC-A KO and cGKI-deficient mutants suggest that ANP- and NO-dependent, cGMP/cGKI mediated vasorelaxation contributes but is not essential to the regulation of basal, chronic blood pressure. This hypothesis is further supported by recently published results (Michael et al. 2008). These authors inactivated the leucine zipper of cGKIα. The mutated cGKIα interacted poorly with RhoA and showed a higher basal kinase activity than the wild type enzyme. The mutant mice developed within 10 weeks an elevated blood pressure, but had a normal live expectancy. These results are partially contradicted by studies that showed that vascular smooth muscle reconstitution of cGKIα or cGKIβ normalized the elevated blood pressure of $cGKI^{-/-}$ mice (Weber et al. 2007). A potential explanation of the discrepant results could be that active cGKIα that is not localized to its appropriate targets such as RGS2 and MYPT1 phosphorylates proteins not modified in wild type cells and does not phosphorylate its target proteins. These different modified proteins then induce hypertension.

3.3 Vascular Remodeling

In addition to vasodilatation, NO/cGMP signalling has been reported to be involved in the development of vasculo-proliferative disorders, such as restenosis and atherosclerosis. The analysis of transgenic mice showed that NO can both promote (Chyu et al. 1999; Detmers et al. 2000; Kuhlencordt et al. 2001a; Ozaki et al. 2002; Sennlaub et al. 2001; Shi et al. 2002; Tolbert et al. 2001) and inhibit (Chen et al. 2001; Knowles et al. 2000; Koglin et al. 1998; Kuhlencordt et al. 2001b; Moroi et al. 1998; Rudic et al. 1998) pathological vascular remodelling. These findings might explain why NO-generating drugs have not been reported to limit the progression of atherosclerosis or restenosis in humans (Lablanche et al. 1997). The opposing effects of NO on vascular remodelling might depend on the spatiotemporal profile of its production (cellular source, time and quantity) and are probably mediated by different cellular and molecular mechanisms (Poon et al. 2003).

A key process in vascular remodelling is the phenotypic modulation of VSMCs from contractile to synthetic phenotype (Berk 2001; Dzau et al. 2002; Owens et al. 2004; Schwartz et al. 1995). High concentrations of NO inhibit VSMC growth in vitro, even in the absence of cGKI (Hofmann et al. 2006), whereas the "growth-promoting" effect of cGMP was absent in primary VSMCs isolated from cGKI-deficient mice (Weinmeister et al. 2008). Recent evidence shows that the "growth-promoting" effect of cGKI in primary smooth muscle cells is caused by an increased adhesion of the wild type VSMCs to the substrate during the establishment of the cell culture (Weinmeister et al. 2008). These investigations demonstrated also that cGMP slightly inhibited the growth of cGKI-positive but not cGKI-deficient VSMCs, if the cells were passaged several times (Fig. 3).

Most likely, the cGKI-dependent regulation of "cell growth" is mediated by affecting gene expression (Eigenthaler et al. 1999; Lincoln et al. 2001; Pilz and Casteel 2003). VSMCs can reversibly change their phenotype from a differentiated, "contractile" phenotype with high levels of smooth muscle (SM)-specific gene expression to a de-differentiated, "synthetic" phenotype with reduced levels of SM-specific gene expression (Lincoln et al. 2006; Owens et al. 2004). This phenotypic switching plays an important role in the development of vascular diseases: in acutely injured blood vessels, e.g. after balloon angioplasty, VSMCs proliferate and migrate from the medial layer of the vessel wall contributing to a "neo-intimal" layer, and the majority of SM-like cells found in atherosclerotic plaques appear to represent de-differentiated VSMCs originating from the medial layer (Lincoln et al. 2006; Owens et al. 2004). The regulation of VSMC phenotypic switching is complex and mediated by multiple factors, but it is clear that de-differentiated VSMCs are a major cell type responsible for the generation of vascular lesions (Kawai-Kowase and Owens 2007). Primary VSMCs cultured in vitro undergo changes similar to those observed in neo-intimal smooth muscle-like cells, including phenotypic de-differentiation, decreased expression of SM-specific genes, and loss of cGKI (Boerth et al. 1997; Cornwell et al. 1994; Lincoln et al. 2006). When these de-differentiated, cGKI-deficient VSMCs are transfected with expression vectors encoding cGKI to restore physiologic levels of cGKI activity, the cells develop a more contractile phenotype,

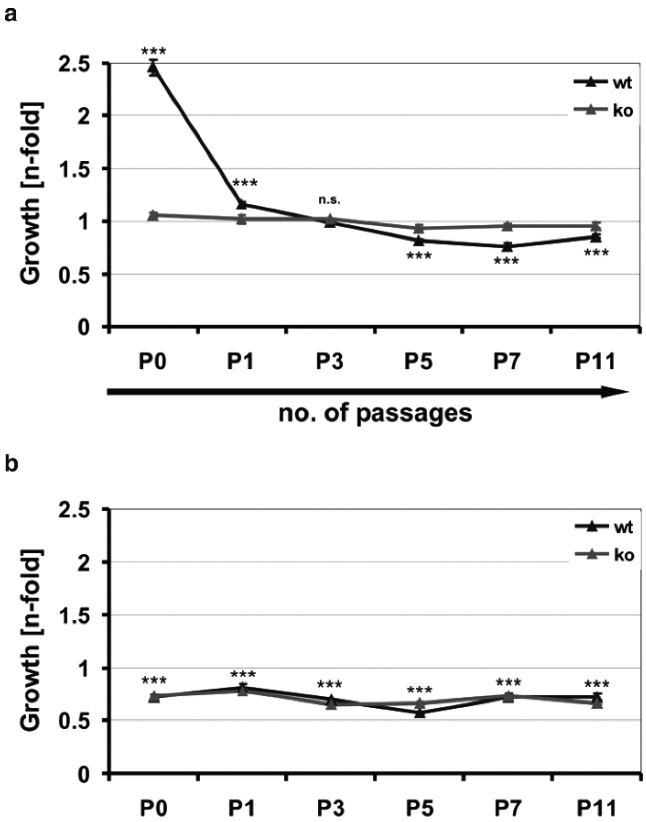

Fig. 3 Growth properties of cultured VSMCs. Growth performance of primary (P0) up to passage 11 (P11) VSMCs in response to 8-Br-cGMP and 8-Br-cAMP. Cell number was measured by the tetrazolium salt MTS (MTS assay). **a** Cells were treated with 0.1 mM 8-Br-cGMP, or **b** 0.1 mM 8-Br-cAMP, respectively. Growth was normalized to control (untreated cells) (>1 = increased growth, 1 = no growth, <1 = decreased growth in comparison to control). **a** Growth is significantly increased in primary wt cells in response to 8-Br-cGMP (***, $p < 0.001$), whereas growth is significantly reduced in subcultured (>P5) cells. Treating ko cells with 8-Br-cGMP has no effect on growth. **b** 8-Br-cAMP significantly inhibits growth in wt and ko cells. n = 5–8 wells. Error bars represent SEM

increase expression of SM-specific genes such as SM-myosin heavy chain (SM-MHC), SM-α-actin (SMA), and calponin, and reduce production of extracellular matrix proteins and growth-related genes. These results suggest that cGKI may contribute to the switch from synthetic/proliferative to contractile VSMCs.

Most SM-specific promoters, including the SM-MHC, SMA, and calponin promoter, contain multiple CArG (CC(AT)6GG) elements recognized by the ubiquitously expressed serum response factor (SRF) (Liu and Olson 2006). Expression of these genes depends on the interaction of SRF with multiple cofactors, including the cysteine-rich LIMonly proteins CRP1 and CRP2/smLIM (smooth muscle LIM protein) (Liu and Olson 2006). Recently, a new member of the CRP family was

identified through a yeast two hybrid screen that used cGKIβ as bait (Huber et al. 2000). This protein was independently cloned from a rat brain and human intestinal cDNA library (Karim et al. 1996; Okano et al. 1993) and was named "CRP2" and recently been renamed to "CRP4" (Zhang et al. 2007). CRP4 is phosphorylated by cGKI in vitro and in vivo (Schmidtko et al. 2008; Zhang et al. 2007). cGKI was required for maintaining SM-specific gene expression in several differentiated smooth muscle cell lines, and enhanced SRF- and GATA6-induced differentiation of pluripotent embryonal cells into smooth muscle cells (Zhang et al. 2007). CRP4 was associated with SM-specific promoters and mediated positive transcriptional effects of cGKI on SM-specific gene expression. These results establish for the first time a plausible link for the effect of cGKI on gene expression.

The above discussed results led to the suggestion that cGMP/cGKI signalling might also contribute to the phenotypic modulation and growth of VSMCs during pathological vascular remodelling in vivo (Sinnaeve et al. 2002; Zhang et al. 2007). To test this hypothesis, the consequences of postnatal smooth muscle-specific cGKI deletion were studied in wild type and ApoE-deficient mice. Smooth muscle-specific deletion of cGKI did not affect restenosis in response to carotid ligation in normo-lipidemic wild type mice nor in apoE-deficient mice (Lukowski et al. 2008). Continuous elevation of cGMP by sildenafil had no effect on neointima proliferation after carotid ligation. Furthermore, VSMC-specific deletion of cGKI had no effect on vascular remodelling after wire induced removal of the endothelial cell layer. These studies are consistent with the conclusion that the role of the smooth muscle cGMP/cGKI pathway is negligible in vascular remodelling in vivo under normo-lipidemic condition. An identical conclusion has been published by Sinnaeve and coauthors (Sinnaeve et al. 2002). Gene transfer of the wild type cGKIβ isoform had no effect on restenosis in vivo. These results contrast those reported above and suggest that cGMP/cGKI signalling is not critically involved in vascular restenosis in vivo. Further experiments with different cGKI-deficient mouse models and cultured VSMCs are required to decipher the exact effect of cGMP/cGKI on vascular smooth muscle differentiation/dedifferentiation.

4 cGKII

cGK type II was originally identified and cloned from intestinal specimens (Lohmann et al. 1997). Deletion of the *prkg2* gene revealed a number of interesting phenotypes including behaviour and clock effects, intestinal water secretion, bone growth and renal effects.

4.1 Neuronal Effects of cGKII

cGKII, a signal transducer of NO/cGMP, is widely distributed throughout the mammalian brain including regions thought to be related to emotional behaviour, e.g. cortex, hippocampus, the amygdala and midbrain raphe nuclei (el-Husseini et al.

1995; Werner et al. 2004). cGKII is involved in the control of anxiety-like behaviour and the behavioural effects of ethanol (Werner et al. 2004). Wild type and cGKII deficient mice showed marked differences in their ethanol consumption in a free choice-test without a general difference in place preference. When given free access to two bottles filled with water and ethanol, respectively, cGKII$^{-/-}$ mice consumed significantly more ethanol over the course of 6 days, and this effect, again, was observed for two different genetic backgrounds.

Many behavioural responses of animals (e.g. feeding, drinking and locomotor activity) and the underlying neuro-humoral activities are organized in circadian rhythms. Both cGKI and cGKII have been reported to be expressed in subpopulations of SCN neurons (el-Husseini et al. 1995; Oster et al. 2003; Revermann et al. 2002). Mice with an inactivated cGKII gene displayed a virtually normal spontaneous circadian rhythm and unaltered expression patterns of the clock genes *mPer1* and *mPer2* supporting the view that the circadian clock is still intact in the absence of cGKII (Oster et al. 2003). Disruption of the cGKII gene, however, resulted in an impaired light-induced entrainment of the circadian clock within a definite time window. Compared to wild type mice, the phase delay of the clock induced by a light pulse applied at circadian time (CT) corresponding to early night, CT14, was reduced by nearly 50%. The phase advance of the clock induced by a light pulse applied at CT22, corresponding to late night, was not affected in cGKII knockout mice (Oster et al. 2003). At the molecular level, cGKII knockout mice displayed marked differences to wild type mice in light induction of two clock genes during the early period of the night: induction of *mPer1* was enhanced and induction of *mPer2* was strongly reduced. The absence of cGKII did not affect light induction of these genes during late night. In contrast to the results obtained with cGKII-deficient mice, pharmacological studies have placed the cGKs into the signalling pathway for phase advances (Mathur et al. 1996; Weber et al. 1995), and a recent in vitro study reported an essential role of cGKII in the progression of the circadian cycle (Tischkau et al. 2004). The reason for these discrepancies are not clear, but may be explained in part by effects of inhibitors not related to cGKII-inhibition as well as by differences in the experimental systems used, e.g. slice cultures versus whole animals. Furthermore, a function of cGKI, which is also expressed in the SCN (Revermann et al. 2002), cannot be excluded. Conditional knockout mice lacking cGKI in the SCN did not reveal a significant effect on circadian rhythmicity.

4.2 Peripheral Effects of cGKII

4.2.1 Intestine

cGKII is an important regulator of gastrointestinal secretion (Pfeifer et al. 1996). cGKII is located in the secretory epithelium of the small intestine and stimulates chloride and water secretion possibly through phosphorylation of CFTR (Vaandrager et al. 1998). cGKII increased Na^+-absorption in the small intestine

by inhibition of the Na^+/H^+ exchanger 3 (NHE3) through interaction with the G-kinase anchoring protein NHERF2 (Cha et al. 2005; Vaandrager et al. 2000). Stimulation of the cGMP signalling cascade by toxins causes diarrhoea as the *Escherichia coli* heat stable toxin (STa) and guanylin activate the GC-C and thereby increase water secretion in the small intestine. Correspondingly, STa- did not induce diarrhoea in cGKII knockout mice (Pfeifer et al. 1996).

4.2.2 Kidney

cGMP affects blood pressure not only by directly regulating the vascular smooth muscle tone but also by regulation of the renin and aldosterone secretion (Kurtz et al. 1998; MacFarland et al. 1991; Nikolaev et al. 2005). Renin secretion is enhanced by NO through cGMP-dependent inhibition of cAMP hydrolysis mediated by PDE 3 (Kurtz et al. 1998). In contrast, ACTH-dependent aldosterone secretion is inhibited by ANP through cGMP-dependent stimulation of cAMP hydrolysis mediated by PDE2 (MacFarland et al. 1991; Nikolaev et al. 2005). In addition, it was reported that cGMP analogs reduced renin secretion from isolated kidney or juxtaglomerular cells (Henrich et al. 1988; Schricker and Kurtz 1993). Kidney expresses both isozymes of cGK. cGKII is localized together with storage granules in juxtaglomerular cells (Gambaryan et al. 1996). Renin secretion from juxtaglomerular cells was increased in cGKII-deficient mice (Wagner et al. 1998) suggesting that cGMP has a dual role in the regulation of renin secretion. cGKII has been detected in rat and murine zona glomerulosa cells of the adrenal gland (Gambaryan et al. 2003) and might regulate aldosterone secretion. The overexpression of cGKII in rat zona glomerulosa cells enhanced the production of aldosterone (Gambaryan et al. 2003). Likewise, a low salt diet activating the aldosterone system enhanced expression of cGKII (Gambaryan et al. 2003). In contrast, cGKII deletion did not alter basal or low salt stimulated aldosterone secretion in mice. So far cGKII deletion has not affected resting blood pressure. Therefore, it is unlikely that the above reported findings are relevant for the overall blood pressure regulation in resting mice and other animals.

4.2.3 Bone Growth

The endochondral ossification of bones is stimulated by the C-type natriuretic peptide (CNP) (Chusho et al. 2001). Overexpression of CNP rescued achondroplasia, which was induced by a defect in fibroblast growth factor receptor 3 signalling (Yasoda et al. 2004). cGKII knockout mice are dwarfs, which develop short bones caused by a defect in the endochondral ossification at the endochondral plate (Pfeifer et al. 1996; Talts et al. 1998). This defect was not rescued by CNP overexpression indicating that cGKII is essential for endochondral bone development (Miyazawa et al. 2002). Interestingly, the regulation of body size was also impaired in the cGMP kinase deficient nematode *egl4* (Nakano et al. 2004). Furthermore, cGKII-deficient rats exhibited an expanded growth plate and impaired bone healing. The

observed accumulation of postmitotic but nonhypertrophic cells in these rats might be caused by induction of the transcription factor Sox9 and decreased phosporylation of GSK3β leading to hypertrophic differentiation of chondrocytes (Chikuda et al. 2004; Kawasaki et al. 2008; Zhao et al. 2005).

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cGK Substrates

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Abstract Signalling of cGK (cGMP-dependent protein kinases) are mediated through phosphorylation of specific substrates. Several substrates of cGKI and cGKII were identified meanwhile. Some cGKI substrates are specifically regulated by the cGKIα or the cGKIβ isozyme. In various cells and tissues, different cGK substrates exist that are essential for the regulation of diverse functions comprising tissue contractility, cell motility, cell contact, cellular secretion, cell proliferation, and cell differentiation. On the molecular level, cGKI substrates fulfill various

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cellular functions regulating e.g. the intracellular calcium and potassium concentration, the calcium sensitivity, and the organisation of the intracellular cytoskeleton. cGKII substrates are involved e.g. in chloride transport, sodium/proton transport and transcriptional regulation. The understanding of cGK signalling and function depends strongly on the identification of further specific substrates. In the last years, diverse approaches ranging from biochemistry to genetic deletion lead to the identification and establishment of several substrates, which raised new insights in the molecular mechanisms of cGK functions and elucidated new cellular cGK functions. However, the analysis of the dynamic signalling of cGK in tissues and cells will be necessary to discover new signalling pathways and substrates.

Keywords: cGK signalling · cGK substrates · cGKI · cGKII · Phosphorylation

Abbreviations

aa, amino acids; ANP, atrio-natriuretic peptide; angiotensin II, ATII; BNP, brain-natriuretic peptide; BK_{Ca}, Ca^{2+}-activated maxi-K^+ channel; CNP, C-type natriuretic peptide; cGK, cGMP-dependent protein kinases; CFTR, cystic fibrosis transmembrane conductance regulator; CRP, cysteine-rich LIM-only protein; FHOD1, forming homology domain protein 1; GC, guanylyl cyclase; GPIbα, glycoprotein Ibα; GluRI, glutamate receptor I; hSERT, human serotonin transporter; IP_3RI, inositol 1,4,5-trisphosphate receptor I; IRAG, IP_3RI-associated cGMP kinase substrate; LASP1, LIM and SH3 domain protein1; NHE3, Na^+/H^+ exchanger 3; NP, natriuretic peptide; NPRA, natriuretic peptide redeptor A; MLCP, myosin light chain phosphatase; MYPT, regulatory myosin phosphatase targeting subunit; pGC, particulate guanylyl cyclase; PDE, phosphodiesterases; Rap1GAP2, Rap1-specific GTPase-activating protein 2; RGS, regulator of G protein signalling; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; sGC, soluble guanylyl cyclase; SM, smooth muscle; SMA, smooth muscle-α-actin; SM-MHC, smooth muscle-myosin heavy chain; StAR, steroidogenic acute regulatory protein; SMTNL1, smoothelin-like protein 1; TFII-I, transcription factor II-I; thromboxan receptors, TP; TRIM39, tripartite motif protein 39; VASP, vasodilatator-specific protein.

1 Introduction

Substrates of cGMP-dependent protein kinases (cGK substrates) are essential elements of the NO, NP/cGMP signal transduction cascade. The identification of these signal transduction elements is important for the understanding of the cGK signalling mechanism on the molecular level and the physiological effects in the diverse tissues. Therefore, the diverse substrates that were found by different approaches (see below) substantiated the knowledge of the cGK effects mediating diverse processes including smooth and cardiac muscle relaxation and proliferation, inhibition of platelet function and hippocampal learning (Hofmann et al. 2006). The

three different cGKs cGKIα, cGKIβ, and cGKII mediate specific substrate recognition and targeting via their distinct aminoterminal leucine zipper containing regions. Specific substrate interaction is essential for integrity of diverse cGMP signalling pathways (Ammendola et al. 2001; Geiselhoringer et al. 2004; Michael et al. 2008). However, it is a matter of debate whether the diverse signalling pathways of cGKIα and cGKIβ can be physiologically compensated (Weber et al. 2007). Other chapters of this volume describe several functional aspects of cGMP and cGMP kinases. In this chapter, we will give an overview about the analysis and identification of cGK substrates, a summary of the known cGK substrates in diverse tissues, and the phenotypes of deletion mutants of these substrates.

2 Overview of cGK Substrates

Several substrates for cGKI and cGKII were identified in the last years. In this review, we summarized the identified substrates for these both enzymes regarding their up to now established function and localization in tissues and cells. Most of the identified cGK substrates are regulated by cGKI. Many of these cGKI substrates are regulated by both cGKI isozymes. However, some substrates are specifically recognized by cGKIα e.g. regulatory myosin phosphatase targeting subunit 1 (*MYPT1*) or cGKIβ e.g. IP_3RI-associated cGMP kinase substrate (*IRAG*) and transcription factor II-I (*TFII-1*). Only few substrates of cGKII were identified till now, including cystic fibrosis transmembrane conductance regulator (*CFTR*), the transcription factor (*SOX9*), and the glutamate receptor I (*GluRI*). A summary of the cGK substrates including their discovered function were listed in Table 1.

In this review, several aspects of cGK substrates are discussed. Approaches for the identification of cGK substrates will include (a) the characteristics and the identification of substrate peptides and (b) approaches for the biochemical and genetical analysis of cGK substrates and functions. In cGKI substrates and cGKII substrates, the functional role of the diverse substrates will be discussed regarding different tissues and cells. These molecular and physiological aspects of cGKI and cGKII substrates were also summarized in Tables 1 and 2 and in Figs. 1 and 2.

3 Approaches for the Identification of cGK Substrates

3.1 Substrate Peptides

cGMP-dependent protein kinases phosphorylate their substrates at serine/threonine residues located in the specific recognition sequence K/R K/R X S/T. This common sequence motif can be used to search for proposed cGK-substrate sequences in a protein. However, the actual phosphorylation of this proposed phosphorylation site at a given protein has to be confirmed by in vitro and/or in vivo phosphorylation

Table 1 CGK substrates

Tissue/cell type	Substrate	MW (kDa)	cGK isoform	Function of phosphorylation	Reference
Cerebellum	G-substrate	32	cGKI	Protein phosphatase-1 inhibitor, initiation of LTD	Endo et al. 2003
	IP_3 receptor type I	230	cGKI	Stimulation of calcium release from IP_3-sensitive stores	Haug et al. 1999; Wagner et al. 2003
Chondrocytes	Sox9	56	cGKII	Bone/chondrocyte development	Chikuda et al. 2004
GIT (epithelial cells)	CFTR	170	cGKII	Chloride secretion/regulation	Vaandrager et al. 1998
	NHERF2	37	cGKII	NHE3 regulation (inhibition)	Cha et al. 2005
Heart	RGS-4	25–26	cGKI	Mediates anti-hypertrophic effects of locally secreted natriuretic peptides	Tokudome et al. 2008
	Troponin T	33	cGKI	Regulation of muscle contraction	Yuasa et al. 1999
Hippocampus	GluR1	100	cGKII	Surface expression of AMPARs at extrasynaptic site	Serulle et al. 2007
	Septin-3	40	cGKI	Vesicle trafficking	Xue et al. 2004
	RhoA	22	cGKI	Reduced MLC phosphorylation, Vesicle trafficking	Ellerbroek et al. 2003
	VASP	46/50	cGKI	Regulation of the actin cytoskeleton, vesicle trafficking	Butt et al. 1994; Hauser et al. 1999
Kidney	StAR	30/37	cGKII	Basal aldosterone production	Gambaryan et al. 2003
	NHERF2	37	cGKII	NHE3 regulation (inhibition)	Cha et al. 2005
Leydig cells	StAR	30/37	cGKI/II	Cholesterol transfer, steroidogenesis	Andric et al. 2007
Substantia nigra	DARPP-32	32	cGK	Protein phosphatase-1 inhibitor, modulation of glutamate signalling	Nishi et al. 2005
Neutrophils	Vimentin	57	cGKI	Regulation of cytosekeleton	Pryzwansky et al. 1995

(continued)

Table 1 (continued)

Platelets	IRAG	125	cGKIβ	Reduced calcium release from IP_3 sensitive stores	Geiselhöringer et al. 2004; Schlossmann et al. 2000
	IP_3 receptor type I	230	cGKI	Stimulation of calcium release from IP_3-sensitive stores	Haug et al. 1999; Wagner et al. 2003
	PDE5	100	cGKI	Enhanced cGMP degradation	Rybalkin et al. 2002
	Rap1GAP2	90	cGKI	Inhibition of Rap1	Danielewski et al. 2005; Hoffmeister et al. 2008; Schultess et al. 2005
	Thromboxane receptor (TPα)	55	cGKI	Receptor desensitization	Reid and Kinsella 2003
	VASP	46/50	cGKI	Regulation of the actin cytoskeleton, vesicle trafficking	Butt et al. 1994; Hauser et al. 1999
Smooth muscle	BK_{Ca}	130	cGKI	Membrane hyperpolarisation; increased open probability	Sausbier et al. 2000a
	CRP2/4	22.5	cGKI	Mediates cGMP/PKG stimulation of SM-specific gene expression; mediates PKG dependently VSMC phenotype	Chang et al. 2007a; Zhang et al. 2007a
	FHOD1	130	cGKI	Inhibition of VSMC stress fiber formation/migration?	Wang et al. 2004
	IP_3 receptor type I	230	cGKI	Stimulation of calcium release from IP_3-sensitive stores	Haug et al. 1999; Wagner et al. 2003
	IRAG	125	cGKIβ	Reduced calcium release from IP_3 sensitive stores	Geiselhöringer et al. 2004; Schlossmann et al. 2000

Table 1 (continued)

Tissue/cell type	Substrate	MW (kDa)	cGK isoform	Function of phosphorylation	Reference
	MYPT1	130	cGKIα	Inhibition of myosin phosphatase inhibition by rho kinase; decreased calcium sensitization	Wooldridge et al. 2004
	PDE5	100	cGKI	Enhanced cGMP degradation	Rybalkin et al. 2002
	Phospholamban	6	cGKI	Enhanced calcium uptake by the Ca-ATPase Serca	Lalli et al. 1999
	RGS-2/4	24	cGKIα	Inhibition of IP_3 generation, reduced GPCR signaling	Sun et al. 2005; Tang et al. 2003
	RhoA	22	cGKI	Reduced MLC phosphorylation, Vesicle trafficking	Ellerbroek et al. 2003
	SMTNL1/CHASM	60	cGKI	Decreased suppression of MLCK activity	Wooldridge et al. 2008
	Telokin	17	cGKI	Inhibition of MLCK activity	Walker et al. 2001
	Thromboxane receptor (TPα)	55	cGKI	Receptor desensitization	Wikstrom et al. 2008
	TRIM39R	46	cGKI	Cellular homeostasis?	Roberts et al. 2007
	VASP	46/50	cGKI	Regulation of the actin cytoskeleton, vesicle trafficking	Butt et al. 1994; Hauser et al. 1999
Spinal cord	CRP2	22.5	cGKI	Nociceptive processing	Schmidtko et al. 2008
Striated muscle fiber (type 2a)	SMTNL1/CHASM	60	cGKI	Modulation of contractile activity	Wooldridge et al. 2008

Table 2 Phenotypes of murine cGK substrate mutants

Deleted/mutated cGKI substrate	Phenotype	Reference
BK_{Ca} (BK α subunit deficiency)	Abnormal conditioned eye-blink reflex; abnormal locomotion and pronounced deficiency in motor coordination; elevated blood pressure and primary hyperaldosteronism	Sausbier et al. 2004, 2005
CRP2/4	Increased neointima formation following arterial injury; Increased nociceptive behavior in models of inflammatory hyperalgesia, no increase in nociceptive behavior after intrathekal treatment with high dose cGMP analogs	Schmidtko et al. 2008; Wei et al. 2005
MYPT1	Embryonic lethality (before 7.5 dpc)	Okamoto et al. 2005
IRAG (disrupted IRAG-IP_3RI interaction)	Dilated gastrointestinal tract; disturbed gastrointestinal motility; increased weight of stomach, shortened expectancy of life	Geiselhöringer et al. 2004
Phospholamban	Enhanced myocardial performance, shortened time to peak pressure and to half-relaxation; Enhanced left ventricular function	Hoit et al. 1995; Luo et al. 1994
RGS-2	Elevated blood pressure and prolonged vasoconstrictor signaling; increased anxiety responses, decreased male aggression; reduced T–cell proliferation	Oliveira-Dos-Santos et al. 2000; Heximer et al. 2003
IP_3-Receptor Type I	Death in utero; born animals with severe ataxia, tonic and tonic/clonic seizures, epilepsy; death by weaning period (within 21 days after birth); Reduced stimulus contraction in aorta	Matsumoto et al. 1996; Zhou et al. 2008
RGS-4 (lacZ/lacZ)	Increased threshold for pain response, subtle sensorimotor deficits	Grillet et al. 2005
SMTNL1	Decreased response to α-adrenergic agonists, better performance after force endurance training	Wooldridge et al. 2008
Telokin	Decreased MLCP activity in ileal smooth muscle, increased MLC phosphorylation, attenuated Ca^{2+}-desensitization of contractile force by 8-Br-cGMP, slowed rate of force relaxation	Khromov et al. 2006
TP-receptor	Prolonged bleeding times, altered vascular responses to TXA2: Enhanced immune responses to foreign antigens, lymphadenopathy in age, splenomegaly	Kabashima et al. 2003; Thomas et al. 1998
VASP	Enhanced platelet adhesion under physiological and pathophysiological conditions; megakaryocyte hyperplasia; Later onset of hearing during postnatal development	Dazert et al. 2007; Hauser et al. 1999; Massberg et al. 2004

(continued)

Table 2 (continued)

Deleted/mutated cGKI substrate	Phenotype	Reference
Vimentin	Normal development and reproduction, normal neutrophil function and inflammatory response to several models	Moisan et al. 2007
GluRI (AMPA receptor subunit)	Reduced functional AMPA receptors in hippocampal CA1 pyramidal neurons, absence of associative LTP in CA3 to CA1 synapses in adult knockout mice; striatal hyperdopaminergia and 'schizophrenia-related' behavior	Wiedholz et al. 2008; Zamanillo et al. 1999
Sox9	Before mesenchymal condensation (limb buds): complete absence of both cartilage and bone, After mesenchymal condensation (embryos): severe generalized chondrodysplasia	Akiyama et al. 2002
StAR	Males and females show female external genitalia, failure to grow normally, death from adrenocortical insufficiency, loss of negative feedback regulation at hypothalamic-pituitary levels, florid lipid deposits in adrenal cortex and testis; Impaired steroidogenesis, lipid accumulation, gonadal lipid deposits	Caron et al. 1997; Ishii et al. 2002
CFTR (several knockout and mutants)	Severe diseases: abnormal electrophysiological profiles, goblet cell hyperplasia, intestinal obstruction, peritonitis; Blockage of small pancreatic ducts, malabsorption; Failure of effective mucociliary clearance, postbronchiolar hyperinflation of alveoli, parenchymal interstitial thickening with evidence of fibrosis, inflammatory cell recruitment.	Guilbault et al. 2007

studies. A detailed analysis of the substrate recognition sequence, to reveal a more specific recognition sequence motif, used peptide libraries of 12-mer and 14-mer peptides spotted on cellulose paper resulting in highly cGKIα selective peptides. The sequences TQAKRKKSLAMA-amide and TQAKRKKSLAMFLR-amide, with K_m values for cGKIα of 0.7 and 0.26 μM and V_{max} values of 11.5 and 10 μmol min^{-1} mg^{-1}, respectively, display a high specificity for this enzyme. Furthermore, replacing the phosphate acceptor residue, serine with alanine in TQAKRKKSLAMA-amide resulted in the highly cGMP-kinase Iα selective inhibitor peptide TQAKRKKALAMA-amide, with inhibitor constants for cGKIα and cAMP-kinase of 7.5 and 750 μM, respectively (Dostmann et al. 1999; Tegge et al. 1995).

Substrate peptide binding of the catalytic cGK domain is mediated by a C-terminal PKG region. Mass-Spectrometry-based hydrogen/deuterium exchange

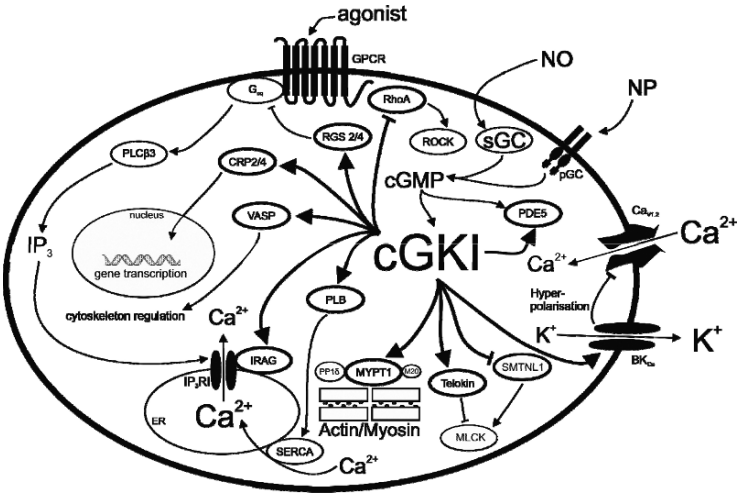

Fig. 1 Signalling mechanisms of cgki substrates in (smooth/striated) muscle cells

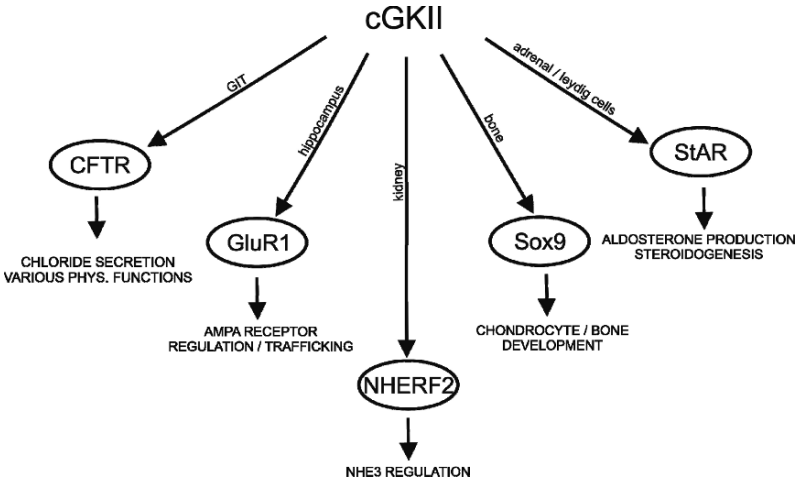

Fig. 2 Signalling mechanisms of cGkII substrates in various tissues and cells

experiments revealed a specific disclosure of the substrate-binding region of holo-cGKI comprising aa 475–655, revealing a new substrate-recognition and kinase-activation process of cGKI (Alverdi et al. 2008).

3.2 Substrate Proteins

Diverse approaches lead to the identification of cGK substrates and their signalling mechanisms. The phosphorylation of a protein in vitro and in vivo is an essential

parameter for the identification of cGK substrate. The incorporation of radioactive phosphate in kinase assays is therefore an essential tool for substrate analysis. However, these assays are sometimes misleading as crossactivation of kinases also occur. Therefore, tissue lysate extracts and heterologous expression systems in combination with phosphosite-specific mutants were used to identify substrates. These approaches were successful for a variety of cGKI substrates including *IRAG*, the vasodilatator specific protein (*VASP*), *RhoA*, *MYPT1* and the regulator of G protein signalling 2 (*RGS2*) and cGKII substrates including *CFTR* (Hofmann et al. 2006; Huber et al. 2000; Schlossmann et al. 2000; Surks et al. 1999; Tang et al. 2003).

Furthermore, phospho-specific antibodies are a versatile tool for the identification of kinase substrates. Therefore, an approach for the identification of in vivo cGK phosphorylation sites is the development of phosphopeptide specific antibodies. The phosphorylation at Ser239 in *VASP* is an established marker for the analysis of cGK-specific phosphorylation in tissues and cells (Oelze et al. 2000; Smolenski et al. 1998). Further phosphospecific antibodies are directed e.g. against *IRAG-S683* and *IRAG-S696* (Antl et al. 2007), *RhoA-S188* (Michael et al. 2008), *MYPT1-T695* (Kitazawa et al. 2003) and *SMTNL1-S301* (Wooldridge et al. 2008).

The stable interaction of cGK with its substrates can be identified in biochemical approaches with affinity chromatography and co-immunoprecipitations using tissue lysates and heterologous expression systems. These approaches revealed also the specificity of the cGKIα and cGKIβ for distinct substrates. *MYPT1* interacts specifically with its C-terminally located coiled-coil-region including AA888–928 to a N-terminal leucine zipper region of cGKIα. Interestingly, mutations in the MYPT1 leucine zipper interrupts MYPT1 homodimerization and the MYPT1-cGKIα interaction (Given et al. 2007; Lee et al. 2007). *RGS2*, a regulator of Gq, binds to cGKIα in smooth muscle cell lysate. NO and cGMP treatment of smooth muscle cells enhances RGS2 membrane translocation (Tang et al. 2003). The N-terminal leucine zipper region of cGKIβ binds to AA152–184 of *IRAG* (Ammendola et al. 2001; Schlossmann et al. 2000). The interaction of cGKIβ-IRAG at these sites is steady and not affected by the presence of cGMP. IRAG targets cGKIβ to the endoplasmic reticulum. This targeting prevents the cGMP-induced nuclear translocation of cGKIβ in baby hamster kidney (BHK) cells (Ammendola et al. 2001; Casteel et al. 2008). However, the C-terminal fragment of cGKI, also named cGKIγ, that lacks the N-terminal leucine zipper domain might be the nuclear form of cGKI (Sugiura et al. 2008). The interface of IRAG-cGKIβ involves electrostatic residues. The mutation of cGKIβ to D26K and E31R in the leucine zipper region disturbed the IRAG-cGKIβ interaction. Vice versa, mutation of positive charged amino acids in the cGKIβ-interacting motif of IRAG strongly affected the IRAG-cGKIβ interaction (Ammendola et al. 2001; Casteel et al. 2005). The interaction of cGKIβ with IRAG is well established in heterologous expression systems and in homologous systems comprising several smooth muscle tissues and platelets (Antl et al. 2007; Geiselhoringer et al. 2004). *TFII-1* is a general transcriptional regulator, which interacts specifically through its R4 helix-loop-helix-motif (AA 491–628) with cGKIβ but not with cGKIα in heterologous expression systems. cGKIβ-TFII-1 interaction is largely cGMP-independent. cGKIβ enhanced TFII-1 transactivation of a serum-response element in COS7-cells (Casteel et al. 2005, 2002).

Interaction analysis via the yeast two-hybrid system has been a versatile tool for the identification of several cGK substrates. Specific interaction partners were found in several approaches using this method. The specific interaction sites of *IRAG* with cGKIβ were identified by the yeast two-hybrid approach, using a smooth muscle cDNA library and specific cDNA constructs of cGKI and IRAG (Ammendola et al. 2001; Schlossmann et al. 2000). The identification of cysteine-rich LIM-only protein, *CRP2* interaction with cGKIα and cGKIβ was detected by the two-hybrid system, using a cDNA library from smooth muscle and from spinal cord (Huber et al. 2000; Schmidtko et al. 2008). *MYPT1* interaction with cGKIα was detected in several approaches using cDNA libraries (Surks and Mendelsohn 2003; Surks et al. 1999). Thereby, essential cGKIα -binding domains of MYPT1 were mapped to COOH terminal domain, which contains also a leucine zipper. The forming homology domain protein *FHOD1* (Wang et al. 2004) interacted with cGKIα in a yeast two-hybrid screen using aortic smooth muscle cDNA. FHOD1 binds to Rho-family GTPases and regulates cytoskeletal processes and gene expression. Furthermore, the tripartite motif protein, *TRIM39* from vascular smooth muscle interacts with cGKI in the yeast two-hybrid approach indicating a regulation of cellular homeostasis (Roberts et al. 2007). Natriuretic peptide receptor, *NPRA* interacted with cGKI in a yeast two-hybrid screen of a human cardiac cDNA library. ANP/cGMP-dependent phosphorylation of NPRA might translocate cGKI to the plasma membrane and thereby induce compartmentalization of cGMP-dependent processes. *Troponin T* interaction with cGKIα was found using a skeletal and cardiac muscle cDNA. This binding was confirmed in heterologous expression systems and by in vitro interaction studies with bacterial expressed fusion proteins (Yuasa et al. 1999). The *male germ cell specific protein* was also detected in a two-hybrid screen with cGKIα as bait (Yuasa et al. 2000).

Phosphoproteomic approaches (e.g. platelet) are very attractive to analyse the whole kinome in a given cell or tissue. Several approaches were undertaken, e.g. in platelets (Marcus et al. 2003). However, some limitations of these approaches have to be concerned. If theses approaches are combined with two-dimensional electrophoresis, only a selection of proteins is detectable because of the limited solubilization of membrane proteins and the limited separation of proteins with molecular weight of more than 100 kDa. New approaches to analyze the platelet phosphoproteome, couple the enrichment of phosphopeptides with immobilized metal-ion exchange chromatography and strong cation-exchange chromatography with mass spectrometry. These approaches yielded several promising candidates which might be involved in specific signalling of cGK in platelets including glycoprotein Ibα (*GPIbα*) (Zahedi et al. 2008). Further analysis of these candidates will reveal the specificity and applicability of this approach for the detection of specific cGK substrates.

The inactivation or mutation of cGK substrates in vivo is a versatile tool to analyse the physiological and pathophysiologicsl role of these proteins. Several murine mutants of cGKI and cGKII substrates were generated via targeted deletion in the last years. These substrate mutants will be discussed in Sects. 4 and 5. A summary of these mutants including their phenotypes is given in Table 2.

4 cGKI Substrates in Diverse Tissues and Cells

4.1 Substrates in Smooth Muscle

4.1.1 Vascular and Tracheal System

Vascular relaxation by cGKI is mediated through lowering of intracellular calcium or by calcium desensitation. These processes are initiated by cGKIα and cGKIβ specific signalling pathways. Lowering of intracellular calcium is induced (a) by the trimeric complex consisting of IRAG, IP_3RI, and cGKIβ (Schlossmann et al. 2000) and (b) by modulation of depolarization induced increases in cytosolic Ca^{2+} (Fritsch et al. 2004). The calcium release channel, IP_3RI plays a prominent role for hormone-stimulated contraction in adult and developing (P15) aortic smooth muscle (Zhou et al. 2008). cGKI affects K^+-induced contraction not by phosphorylation of the IP_3RI but most likely by phosphorylation of the L-type Ca^{2+} channel (Mery et al. 1991; Yang et al. 2007). Culturing of vascular smooth muscle cells from thoracic aorta revealed that IP_3RI does not affect smooth muscle differentiation. IP_3RI -deficient mice show cerebellar ataxia (see Sect. 3.4). These mice die within 21 days after birth, presumably resulting from its smooth muscle phenotype. IP_3RI is phosphorylated by cGKI in vascular smooth muscle (Cavallini et al. 1996; Komalavilas and Lincoln 1994, 1996). However, it was suggested that cGMP-dependent phosporylation might not decrease but rather enhance the calcium release activity of the IP_3RI in smooth muscle (Wagner et al. 2003). *IRAG* targets cGKIβ to the endoplasmic reticulum and additionally binds with its coiled-coil-domain to the IP_3RI. The phosphorylation of *IRAG* suppresses the hormone-induced calcium release via the IP_3RI and thereby induces relaxation of the vascular smooth muscle. Mice with a targeted mutation of the N-terminal part of the coiled-coil-domain of IRAG loose the ability to interact with the IP_3RI (Fritsch et al. 2004). This loss of interaction prevents modulation of Ca^{2+} release by cGKIβ. Hence, the NO/cGMP-mediated vascular smooth muscle relaxation of hormone-induced contraction is attenuated in IRAG mutant mice. In contrast, the NO/cGMP-dependent relaxation of depolarization-induced contraction is not affected in IRAG mutant mice.

Upon cGMP stimulation, cGK is able to enter the nucleus and to modulate transcriptional activity in some cellular systems, including fibroblasts, osteoblasts, neutrophils, and neurons (Broderick et al. 2007; Gudi et al. 2000, 1997; Wang et al. 1999; Wyatt et al. 1991). Some cGK substrates e.g. *CRP2*, recently renamed as *CRP4* to prevent confusion with the smLIM protein CRP2, are involved in the nuclear and transcriptional activity of cGKI (Zhang et al. 2007a) (see below). The nuclear translocation can be prevented by the association of cGK with interacting proteins. cGKIβ stably associates with *IRAG*, independent of cGMP (Ammendola et al. 2001; Casteel et al. 2008). This association is sufficient to inhibit the entry of cGKIβ into the nucleus. In the absence of IRAG or in the presence of an IRAG mutant deficient in cGKIβ-binding, a nuclear localisation of cGKIβ can

be induced upon cGMP addition in BHK cells mediating transcriptional activity. cGMP-dependent phosphorylation of IRAG does not affect the interaction with cGKIβ and is hence not sufficient to induce translocation of cGKIβ into the nucleus.

The calcium activated maxi potassium channel (BK_{Ca}) is a dominant factor for the regulation of the membrane potential. Deletion of the murine BK_{Ca} channel results in slightly enhanced vascular smooth muscle tone and hypertension. This hypertensive effect is a result of vasoconstriction and hyperaldosteronism (Sausbier et al. 2005) (see also Sect. 4.4). Interestingly, cholinergic bronchoconstriction and isoprenaline-mediated airway relaxation was enhanced in BK_{Ca}-deficient mice. This might be caused by the fact that the cGMP signalling pathway (e.g. the expression of cGKI and IRAG) was upregulated in mutant tracheal muscle (Sausbier et al. 2007).

Phosphorylation of *phospholamban* by cGKI that abrogates the inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase could be a cGKI pathway, which induces NO/cGMP relaxation. However, the deletion of the murine phospholamban gene did not affect blood pressure and only marginally affected NO/cGMP-dependent relaxation of vascular smooth muscle strips (Lalli et al. 1999).

Thromboxan A2 signals via the thromboxan receptors, TPα and TPβ. TPα is phosphorylated at Ser331 by cGKI affecting signalling via phospholipase Cβ (Reid and Kinsella 2003). Furthermore, TPα signalling via $G_{12/13}$ and RhoA is affected by NO in primary vascular smooth cells (Wikstrom et al. 2008).

RGS proteins (regulator of G-protein signalling) contain a 120 aa RGS domain, which mediates direct binding to Gαq and Gαi. Additional coupling to other interaction partners was observed for most RGS proteins. RGS couples also to adenylyl cyclases, adrenergic and muscarinergic receptors. *RGS2* is located in diverse tissues comprising smooth muscle, heart, bone, immune system, olfactory epithelium, and brain (Bansal et al. 2007). RGS2 is phosphorylated by cGKIα at Ser46 and Ser64. Activation of RGS2 via cGKIα increases the inactivation of Gαi and Gαq and inhibits thereby thrombin signalling. Targeted deletion of RGS2 results in a pleiotropic phenotype including hypertension, reduced inflammation and reduced neurotransmitter release in the hippocampal CA1 neurons (Heximer et al. 2003; Oliveira-Dos-Santos et al. 2000). There is evidence that a decrease of RGS2 expression and/or function is involved in the pathology of human hypertension (Semplicini et al. 2006). RGS2-deficient mice developed renovascular abnormalities with increased responsiveness to vasopressin (Zuber et al. 2007) (see also Sect. 3.5). Nitric oxide might regulate the blood pressure via vascular RGS2 during the day (inactive phases of mice), whereas the sympathetic nervous system might be the dominant regulating factor of blood pressure via RGS2 during the active phase (Obst et al. 2006; Sun et al. 2005; Tang et al. 2003; Tank et al. 2007).

The sustained phase of smooth muscle contraction depends on active RhoA. Active RhoA translocates to the plasma membrane, where it activates Rho kinase that phosphorylates the 130 kDa myosin binding subunit MYPT1 of myosin phosphatase. This phosphorylation inhibits the activity of myosin phosphatase. cGKI phosphorylates *RhoA* at Ser188 in freshly dispersed and cultured smooth muscle cells and thereby inactivates RhoA signalling, because this phosphorylation translocates RhoA back to the cytosol (Murthy et al. 2003).

cGKIα phosphorylates a variety of proteins in vascular smooth muscle cells including *MYPT1*. MYPT1 interacts with the leucine zipper region of cGKIα. To test the in vivo effect of cGKIα-specific interactions, the leucine zipper region of cGKIα was mutated in mice (Michael et al. 2008). This mutation resulted in abnormal relaxation of large and resistance blood vessels and increased systemic blood pressure. Targeted deletion of MYPT1 in mice is embryonic lethal before day 7.5 pc, indicating that MYPT1 is essential for embryogenesis (Okamoto et al. 2005). Interestingly, two different myosin phosphatase forms are known that occur during development of smooth muscle: (a) a leucine zipper positive C-terminal region, which allows binding of cGKIα that leads to cGMP-dependent relaxation of smooth muscle and (b) a leucine zipper negative C-terminal region, which is unable to bind cGKIα and is insensitive to cGMP (Payne et al. 2006). MYPT1 is phosphorylated by cGKI at Ser-695, which phosphorylation prevents phosphorylation of MYPT1 by Rho kinase at the inactivating site Thr-696 (Wooldridge et al. 2004).

Signalling via Rho/Rho kinase might be also involved in a mechanism for NO/cGMP dependent effects on proliferation and gene expression. Mechanisms discussed further in this context include the transcriptional nuclear factor of activated T cells (*NFAT*) and *VASP*, an actin-binding protein (Zhuang et al. 2004). The deletion of VASP did not affect the cyclic nucleotide mediated relaxation of vascular and intestinal smooth muscle (Aszodi et al. 1999). In HEK cells, heterologously expressed cGKIβ phosphorylated the *VASP* protein in a cGMP-dependent manner. This phosphorylation did not reduce thrombin-induced calcium response suggesting that VASP phosphorylation by cGKIβ is not involved in reduction of elevated calcium levels (Meinecke et al. 1994). NO and ANP induced cGKI-phosphorylation of *VASP* Ser-239 in vascular smooth muscle and endothelial cells resulted in reorganization of the actin cytoskeleton and enhancement of angiogenesis (Chen et al. 2008, 2004).

The differentiation of smooth muscle cells depends on the expression of smooth muscle specific proteins (e.g. smooth muscle alpha-actin and calponin) determined by smooth muscle specific promoter elements, which are activated by the serum response factor (SRF) and GATA6. cGKI induces the expression of smooth muscle specific proteins. cGKIα and Iβ interacts with its substrate CRP2 (renamed as CRP4) in a complex with SRF (Zhang et al. 2007a). CRP4 is a LIM protein having four zinc fingers. It is phosphorylated by cGKI in vitro and in vivo (Huber et al. 2000; Schmidtko et al. 2008; Zhang et al. 2007a). The third zinc finger of CRP4 is essential for the association with cGKI. CRP4 does not bind itself to smooth muscle specific promotors but enhances the SRF-DNA association, when cGMP/cGKI is present. Downregulation of CRP4 by small interference RNA in the pulmonary artery cell line PAC1 cells prevented the positive effect of cGMP/cGKI on mRNA levels of smooth muscle specific genes e.g. smooth muscle α-actin and calponin (Zhang et al. 2007a).

Phosphodiesterase 5 (PDE5) is phosphorylated by cGKI at Ser92, which increases enzyme activity and cGMP binding affinity (Rybalkin et al. 2002). Phosphorylation increases affinity of the PDE5 catalytic site for tadalafil and thereby might

increase the potency and duration of this PDE5 inhibitor. Inhibition of PDE5 by sildenafil is not affected by this modification (Bessay et al. 2008; Corbin et al. 2000).

4.1.2 Gastrointestinal Tract

The ternary complex of cGKIβ, IRAG, and IP_3RI is also present in gastrointestinal smooth muscle. IP_3RI is phosphorylated, cGMP-dependently in vivo in gastric smooth muscle cells (Murthy and Zhou 2003). IP_3RI phosphorylation by externally added cGKIα correlated with the inhibition of calcium release from microsomes. The effect of cGKIβ was not tested in this study. However, *IRAG* mutant mice with a deficiency in IP_3RI binding have severely reduced cGMP-induced relaxations and strongly enhanced gastrointestinal passage times (Geiselhoringer et al. 2004). The mutation of IRAG leads to enlarged gastrointestinal organs including esophagus, stomach, colon, and caecum. These IRAG mutant mice exhibit a significant reduction in life time.

Recently, smoothelin-like protein 1 (SMTNL1; also named as CHASM: calponin homology activated in smooth muscle) was identified as cGKI-specific substrate, involved in calcium desensitizaton of intestinal smooth muscle (Borman et al. 2004; Niessen et al. 2005; Wooldridge et al. 2008). SMTNL1 is phosphorylated at Ser301 by cGKI. Mice lacking SMTNL1 revealed that this protein is involved in cGMP/cAMP adaptations to exercise (Wooldridge et al. 2008).

Calcium desensitization is more effective in small intestinal than in vascular smooth muscle. Calcium desensitization is prominent in jejunum and ileum. cGKI deletion affected myosin phosphatase activity and contraction. The suggested cGKIα substrates are MYPT1, RhoA (see also Sect. 4.1) and telokin (Hofmann et al. 2006). Calcium desensitization of gastrointestinal smooth muscle might be mediated via telokin, a 17 kDa smooth muscle-specific protein, which represents a fragment of MLCK without a kinase and CaM-binding domain. Analysis of murine telokin-KO mutants suggested that cGMP-dependent phosphorylation of telokin enhanced MLCP activity (Khromov et al. 2006). Interestingly, telokin is not required for filament formation and stability.

4.1.3 Reproduction Apparatus

NO/cGMP signalling is a major pathway mediating penile erection, which is induced by the relaxation of arterial and corpus cavernosum smooth muscle. The deletion of the BK_{Ca} channel in mice suppressed the intra-cavernous pressure that increases upon nerve stimulation, indicating a major role of BK_{Ca} channel activation for penile erection (Werner et al. 2005). Furthermore, sildenafil-induced relaxations were diminished in BK_{Ca} deficient corpus cavernosum indicating a dominant role of the BK_{Ca} channel in NO/cGMP signalling in this tissue (Werner et al. 2008, 2005). Endogenous cGKI enhances BK_{Ca} channel activity during pregnancy in myometrial cells and thus may contribute to the quiescence of the pregnant uterus (Zhou et al. 2000).

4.2 Substrates in Platelets

cGMP-dependent protein kinases mediate the inhibition of platelet aggregation. Several substrates were identified, which are involved in this antiaggregatory effect. A main function of cGKIβ is the inhibition of intracellular increases in calcium elicited by agonists such as thrombin, thromboxan A2, and collagen. An essential substrate for this mechanism is IRAG (Antl et al. 2007). Interestingly, only cGKIβ but not cGKIα is abundant in human platelets underlining the importance of this isozyme for platelet function. Analysis of murine deletion mutants revealed that the interaction of IRAG with the IP_3RI is essential for the NO-mediated prevention of arterial thrombosis. The deletion of VASP induced megakaryocyte hyperplasie and enhanced agonist-induced platelet activation (Hauser et al. 1999; Massberg et al. 2004). VASP deletion affected minimal cAMP- and cGMP-induced inhibition of agonist-induced platelet aggregation. Similarly, cGMP-mediated inhibition of cytosolic calcium concentrations and granule secretion were not influenced by the presence or absence of VASP (Aszodi et al. 1999). Rap1 activation is inhibited by NO/cGMP/cGKI signalling. The Rap1-specific GTPase activating protein, *Rap1GAP2* strongly stimulates the GTPase activity of Rap1 and interacts with 14–3–3 proteins through phosphorlyated Ser9. cGKI-phosphorylation of Rap1GAP2 at Ser7 disrupts 14–3–3 binding and thereby inhibits cell adhesion (Danielewski et al. 2005; Hoffmeister et al. 2008).

4.3 Substrates in Immune and Nociceptive Cells

Treatment of dendritic cells with nitric oxide initially stimulated cGKI-activity and inhibited migration of dendritic cells, probably via NO/cGMP-dependent phosphorylation of VASP. However, long term treatment with nitric oxide inhibited VASP phosphorylation and induced migration of dendritic cells acting as a negative feedback signal (Giordano et al. 2006). Furthermore, VASP phosphorylation might be involved in actin dynamics leading to adhesion and spreading of neutrophils (Lawrence and Pryzwansky 2001). The intermediate filament protein, vimentin co-localized with cGKI in a Ca^{2+}-dependent manner (Pryzwansky et al. 1995).

CRP4 co-localizes with cGKI in the laminas of the murine spinal cord and the dorsal root ganglia. Analysis of CRP4-deficient mice suggested that CRP4 is an important downstream target of cGKI in nociceptive processing (Schmidtko et al. 2008).

4.4 Substrates in Heart

Among more than 30 RGS proteins, RGS2 and RGS4 coupled to Gq proteins have been implicated in cardiovascular physiology. RGS2 is an important factor

regulating NO- and ANP-mediated vasorelaxation. (see Sect. 4.1). RGS2 is strongly expressed in murine coronary artery smooth muscle, but only weakly found in heart myocytes (Tokudome et al. 2008). In contrast, RGS4 is not present in vessels, but is strongly expressed in murine coronary myocytes. Inhibition by RGS proteins is involved in cardiac hypertrophy (Bansal et al. 2007). In this respect, RGS is required for the antihypertrophic effects of locally secreted natriuretic peptides e.g. ANP, which enhanced the binding of RGS4 to cGKIα. Furthermore, binding of RGS4 to Gq is stronger in the presence of ANP. These interactions can be reversed by the addition of the cGMP kinase inhibitor, KT5823 and the natriuretic receptor antagonist, HS142–1. ANP mediated short term effects on RGS4 phosphorylation but also long term induction of RGS4 expression. Interestingly, expression and phosphorylation of RGS4 was reduced in hearts of GC-A knockout mice in comparison to wild type mice. GC-A knockout mice show cardiac hypertrophy, which can be "attenuated" by overexpression of RGS4. Interestingly, RGS4 is upregulated in failing heart due to cardiac myopathy (Owen et al. 2001). In respect to the new approaches identifying cardiac RGS4 function, this overexpression might be compensatory. Studies with RGS4 knockout mice that are viable and exhibit slight central and nociceptive symptoms (Grillet et al. 2005). So far, the cardiac phenotype of these mice was not described.

The extracellular calcium influx via L-type calcium channel is a determining factor for contractility of cardiac cells. Recently, cGKI-dependent phosphorlyation of Cav1.2 (Ser1928) and beta2A (Ser496) subunit was shown. The phosphorylation of the beta2A subunit slightly inhibited calcium channel activity (Yang et al. 2007). This correlates with the results that overexpression of cGKIα in cardiomyocytes enhanced NO/cGMP inhibition but not muscarinic inhibition of the L-type calcium channel (Schroder et al. 2003).

4.5 Substrates in the CNS

cGMP/cGKI signalling is involved several aspects of the CNS including axon guidance, hippocampal and cerebellar learning, behaviour, circadian rhythm, and nociception (Hofmann et al. 2006).

The growth cone guidance and neuronal pathfinding via Sema3A involves the activation of cGKIα. *VASP* phosphorylation could organize this process through remodelling of the actin skeleton (Lebrand et al. 2004).

The role of nitric oxide and cGMP in hippocampal learning and generation of LTP is not clear so far. Nitric oxide and cGMP stimulated the induction of LTP in hippocampal slices. However, the deletion of cGKI and cGKII did not alter the induction of LTP. A refined analysis implied a role of cGKI in the induction of L-LTP (Feil and Kleppisch 2008). RhoA, VASP, and Septin-3 are involved in synaptic assembly and vesicle trafficking in the hippocampus (Wang et al. 2005; Xue et al. 2004). RhoA acts pre- and post-synaptically during potentiation, probably directing the localization of synaptic proteins e.g. GluR1 and synaptophysin. VASP is

localized in synaptic compartments and phosphorylated by cGKI during potentiation. Thereby, VASP might regulate the formation of new synaptic puncta. Septin-3, also called G-septin, belongs to the 40–60 kDa GTPase septin family, which assembles with intracellular filaments and regulate neuronal processes such as polarity and vesicle trafficking. Septin-3 is phosphorylated in situ by cGKI on Ser91 in nerve terminals. Septin-3 phosphorylation induces the translocation of Septin-3 from peripheral membranes to cytosol, thereby might regulate filament assembly.

Signalling of cGMP/cGKI in the cerebellum induces long term depression as a prerequisite for motor learning. G-substrate is located exclusively in cerebellar purkinje cells. It is phosphorylated by cGKIα in vivo at Thr123 and acts as an inhibitor for protein phosphatase 1 and 2a. Thereby, G-substrate alters the phosphorylation state of signalling proteins e.g. AMPA receptors, probably initiating their endocytosis and effecting the initiation of LTD (Endo et al. 2003). IP_3 receptor I is particularly expressed in smooth muscle (see above) and in cerebellar Purkinje cells. This protein is strongly involved in motor learning as murine knockout mutants are particularly affected in motor coordination (Matsumoto and Nagata 1999). The neuronal splice variant of the IP_3 receptor (SII+) is phosphorylated by cGKI in situ at Ser1589 (Haug et al. 1999). However, phosphorylation at Ser1755 but not at Ser1589 induced the release of intracellular calcium after heterologous expression of the neuronal IP_3RI (Wagner et al. 2003). The cerebellar BK_{Ca} channel is predominantly expressed in Purkinje cells. BK_{Ca} mutant mice have normal life expectancy but show motor impairment including an abnormal eye-blink reflex and ataxia. This might result from a severe suppression of Purkinje cell activity and synapses in the BK_{Ca} mutant mice (Sausbier et al. 2004).

The dopamine and cAMP-regulated 32 kDa phosphoprotein (DARPP-32) inhibits protein phosphatase-1 in the substantia nigra and the neostriatum. DARPP-32 is phosphorylated at Thr-34 by cGKI. It integrates dopaminergic and glutaminergic signalling and thereby modulates cognitive processes (Nishi et al. 2005). The human serotonin transporter (hSERT) is involved in presynaptic transmitter homeostasis inactivating released 5-hydroxytryptamine. hSERT is phosphorylated at Thr-276 by cGKI. Different variants of hSERT show varying sensitivity to cGKI, which might correlate with the pathogenesis of obsessive disorders (Zhang et al. 2007b).

4.6 Substrates in the Kidney

The contractility of mesangial cells is decreased by nitric oxide and the atrial natriuretic peptide. Particularly, cGKI-mediated activation of the BK_{Ca} channel was implicated in this process (Sansom and Stockand 1996; Stockand and Sansom 1996, 1998). Interestingly, the deletion of the murine α-subunit of the BK_{Ca} channel leads to hyperaldosteronism (Sausbier et al. 2005) (see also Sect. 4.1) and is involved in K^+-homeostasis (Rieg et al. 2007). Further studies suggested that cGKI-mediated phosphorylation of the human beta1 subunit of BK_{Ca} might be involved in the activation of the BK_{Ca} channel (Kudlacek et al. 2003).

The cGKI-specific phosphorylation of VASP in rat mesangial cells was previously shown in vitro using the cGKI inhibitor, KT5823 (Burkhardt et al. 2000; Gambaryan et al. 2001). Human mesangial cultured cells contain cGKIα, as revealed by RT-PCR. Application of 8-Br-cGMP lowered the intracellular calcium concentration, suggesting a role of cGMP-signalling in contractility of mesangial cells (Wang et al. 2007). Association with and regulation of TRPC4 together with VASP in mesangial cells might cause the decreased calcium response. Furthermore, LIM and SH3 domain protein 1 (LASP1) associates with cGKI in human and mouse mesangial cells (Keicher et al. 2004). Whether these substrates might be involved in migration, proliferation, or differentiation of mesangial cells is unknown.

RGS2 plays a key role in angiotensin II (ATII)-signalling. In Bartter's/Gitelmann syndrome, ATII signalling and vasomotor tone is diminished. The resulting hypotension is caused by sodium and potassium loss and volume depletion, but the genetic background of this disease is not clear so far. Interestingly, RGS2-mRNA and -protein is increased in mononuclear cells (PBM) from BS/GS patients suggesting a role of RGS2 in the control of vascular tone and in the prevention of hypertension (Calo et al. 2004) (see also Sect. 4.1). RGS2 overexpression inhibited ATII-induced aldosterone secretion from adrenal cells (Romero et al. 2006). However, the role of NO/cGMP for renal and/or adrenal RGS2 signalling is not established.

4.7 Substrates in Parasites

The identification of cGKs in parasites stimulated the search for cGK target proteins, which might be involved in parasite infections. (Donald et al. 2002, 2006; Wiersma et al. 2004). Trisubstituted pyrrol inhibitors of cGK lead to the identification of secondary protein kinase targets, which could inhibit the invasion of the parasites.

5 Substrates of cGKII in Diverse Tissues and Cells

cGKII is expressed in a variety of tissues implicating a pleiotropic role of this enzyme. cGKII is involved in intestinal chloride reabsorption, endochondral ossification, kidney function, synaptic plasticity, and steroidogenesis (summarized in the chapter Hofmann et al. this volume). A variety of different substrates were identified regarding these functional aspects.

5.1 Substrates in the Intestine

The activation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is mediated by cGKII (Golin-Bisello et al. 2005; Markert et al.

1995; Pfeifer et al. 1996; Vaandrager et al. 1998, 1997). Specific mutations in CFTR cause cystic fibrosis in humans and mice including severe intestinal and lung defects (Guilbault et al. 2007). CFTR interacts specifically with the N-terminus of cGKII. CFTR activation requires membrane targeting of cGKII via an N-terminal myristoylation site shown in intestinal cell lines. Furthermore, the heat-stable enterotoxin STa- and the cGMP-mediated CFTR translocation were identified in villus enterocytes of rat jejunum (Golin-Bisello et al. 2005). This mechanism might explain that cGKII deficiency leads to resistance against STa (Pfeifer et al. 1996). However, phosphorylation of CFTR by cGKII was experimentally found only in intestinal cell lines (French et al. 1995; Vaandrager et al. 1998).

Sodium absorption in the small intestine is enhanced by cGKII through phosphorylation of NHERF2, thereby inhibiting Na^+/H^+ exchanger 3 (NHE3) (see also Sect. 5.4) (Cha et al. 2005).

5.2 Substrates in Bone

cGKII is a very important regulator of endochondral ossification and bone growth. cGKII lacking mice are dwarfs and exhibit a reduced endochondral ossification (Miyazawa et al. 2002; Pfeifer et al. 1996). These mice show enhanced postmitotic, non-hypertrophic cells. The differentiation of chondrocytes by SOX9 is inhibited in cGKII deficient mice (Chikuda et al. 2004). cGKII phosphorylates GSK3β and inhibits its activity, which is necessary for enhanced hypertrophic chondrocyte differentiation (Kawasaki et al. 2008).

Bone turnover through osteoclast stimulation by NO might involve the phosphorylation of VASP. Thereby, the reorganization of attachment and cytoplasmic proteins might be induced (Yaroslavskiy et al. 2005). However, it is not clear whether cGKI or cGKII is involved in this process.

5.3 Substrates in the Brain

cGKII phosphorylates Ser 845 of the glutamate receptor I (GluR1) and interacts with the C-terminal domain of this receptor. Trafficking of GluRI to extrasynaptic sites and long term potentiation is induced in hippocampal slices. Thereby, GluRI-cGKII interaction might be involved in hippocampal learning (Hardingham and Fox 2006; Lin and Talman 2001; Serulle et al. 2007; Wang et al. 2005). However, LTP was not altered in conventional cGKII deficient mice (Kleppisch et al. 1999). The discrepancy of these results should be further investigated.

5.4 Substrates in the Kidney

Renin secretion from the juxtaglomerular cells might be affected by cGKII which is found in storage granules of JG cells (Wagner et al. 1998). However, the substrates involved in this reduced renin secretion are not characterized so far.

The Na^+/H^+ exchange in the kidney and intestine (see Sect. 5.1) via NHE3 is inhibited by cGMP (Donowitz et al. 2005). The inhibition of NHE3 by plasma membrane bound cGKII is mediated via the G-kinase anchoring protein, NHERF2. NHERF2 interacts with its tandem PDZ domain to NHE3 and cGKII in a ternary complex. Interestingly, cGKI is not able to inhibit NHE3 via NHERF2 (Cha et al. 2005). The reduced Ca^{2+}-reabsorption by cGKII was only observed in freshly prepared collecting tubules, due to cGKII downregulation in primary cultures (Hoenderop et al. 1999). The actual substrates for the inhibition of Ca^{2+}-reabsorption are unknown.

VASP is phosphorylated at Ser-293 by cGKII in primary mammalian renal tubule epithelial cells. This phosphorlyation regulates the actin polymerization within lamellipodia and is associated with the loss of lamellipodial protrusions and cell rounding (Lindsay et al. 2007). The drosophila cGMP-dependent protein kinases are also located in renal tubule epithelial cells. DG1 expression greatly enhanced fluid transport by the tubule in response to exogenous cGMP, whereas DG2P2 expression significantly increased fluid transport in response to the nitridergic (NO-synthase stimulating) neuropeptide, capa-1. Thus, DG1 and DG2 are bona fide cGKs, which have differential roles in epithelial fluid transport as assessed by in vivo studies (Kean et al. 2002; MacPherson et al. 2004). In collecting duct principal cells, aquaporin 2 is phosphorylated in the COOH-terminal tail in a cGMP-dependent manner. It was suggested that cGK was involved in this phosphorylation. This phosphorylation leads to enhanced membrane insertion and therefore water reabsorption (Bouley et al. 2000). The cGK enzyme, which might be involved in this phosphorylation was not identified so far.

5.5 cGKII Substrates in Further Tissues and Cells

Human 6-pyruvoyltetrahydropterin synthase (PTPS) is a cofactor of tetrahydrobiopterin biosynthesis. PTPS-Ser19 is phosphorylated by cGKII in embryonic fibroblasts and thereby inactivated (Scherer-Oppliger et al. 1999). This effect on BH4-biosynthesis might influence either peripheral organ function e.g. in kidney or central functions e.g. catecholamine or serotonin biosynthesis.

Leydig cells of testes activate steroidogenesis in a cGMP-dependent manner. NO treatment of these cells stimulates the phosphorylation of steroidogenic acute regulatory protein (StAR). StAR is a nuclear encoded inner mitochondrial membrane protein exposed to the mitochondrial matrix and exhibits a 37 kDa precursor and 30 kDa mature protein. Steroid synthesis in gonadal and adrenal cells requires the StAR protein for translocation of cholesterol from the cytoplasm to the inner mitochondrial membrane. Interestingly, male and female knockout mice of StAR show

female external genitalia, a growth defect and adrenocortical insufficiency leading to death (Caron et al. 1997). cGKI and cGKII are expressed in the Leydig cells and the activation of cGKs stimulates the production of androgen and progesterone in these cells (Andric et al. 2007).

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Biochemical Detection of cGMP From Past to Present: An Overview

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Abstract Cyclic guanosine monophosphate (cGMP), generated via the guanylate cyclase (GC)-catalyzed conversion from GTP, is unequivocally recognized as crucial second messenger, intimately involved in the regulation of a broad range of physiological processes such as long term potentiation, blood pressure regulation, or platelet aggregation (for review: Hobbs 2000). Since its first identification in rat urine by Ashman and co-workers (1963), various approaches have been conceived and established to quantify cGMP in biological samples, or to detect cGMP as the reaction product of enzymatic assays, allowing the determination of kinetic parameters. These approaches have evolved from laborious handling of small numbers of samples with average sensitivity to highly developed biochemical detection assays allowing the processing of very large numbers of samples. The present article focuses upon the history of biochemical cGMP detection from the pioneering work of the early years to the actual state-of-the-art approaches for the detection of this important biological messenger.

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1 Introduction

Cyclic guanosine monophosphate (cGMP) is generated via the catalytic activity of the enzyme family of guanylate cyclases (GC). This class includes the transmembrane particulate GCs (pGC) like GCA or GCB, which represent receptors for peptide ligands such as ANP, BNP, or CNP (for review: Lucas et al. 2000), as well as the cytosolic receptor for the gaseous messenger nitric oxide (NO) soluble guanylate cyclase (sGC) (for review: Hobbs 2000). Upon activation, both classes of GC catalyze the conversion of guanosine triphosphate (GTP) into the second messenger cGMP, which in turn regulates various effector systems such as ion channels, protein kinases, and phosphodiesterases. The breakdown of cGMP to guanosine monophosphate (GMP) is catalyzed by the enzyme family of phosphodiesterases (PDEs) including PDE2, PDE9, or PDE5 (for review: Bender and Beavo 2006). The latter represents the target for the cGMP elevating drugs Sildenafil (Viagra), Vardenafil (Levitra), and Tadalafil (Cialis) (Neumeyer and Kirkpatrick 2004; Ghofrani et al. 2006). The clinical impact of these drugs and the perspective that further cGMP elevating drugs such as NO-independent sGC activators (Evgenov et al. 2006; Stasch et al. 2006) might offer new therapeutic approaches have attracted broad attention to this scientific field. The biochemical detection of cGMP has therefore become an important prerequisite for both the quantification of tissue cGMP levels upon treatment with drugs such as PDE inhibitors, as well as the characterization of novel drugs using purified GC preparations.

The numerous assays which have been conceived and established over the last four decades to biochemically detect cGMP can be classified into two broad classes whose differences based on their application are, however, fading. The detection of GC catalytic activity in biological samples (e.g., homogenized cells or tissues) has been and is still performed by the addition of labelled substrate such as radioactive GTP. The subsequent separation of substrate and product to estimate GC catalytic activity has been developed from the initial attempts using thin layer chromatography (TLC) to chromatography on alumina column, which is still the basis of most radioactive cGMP assays.

In contrast to this, the approach, which has become most widely accepted for the detection of unlabelled cGMP in biological samples, is the competition of physiological cGMP with an exogenous labelled cGMP derivative for a common binding protein. At first, this approach was performed with undefined cGMP-binding protein homogenates, limiting the sensitivity of this competition-binding assay (Murad et al. 1971). The ground-breaking discovery for the biochemical detection of cGMP that still impacts on the actual assays formats was the development of specific anti-cGMP antibodies by Steiner and co-workers (Steiner et al. 1969, 1972a). Although most assays established within the last ten years are based on the competition of labelled and unlabelled cGMP for the specific binding sites of anti-cGMP antibodies, the approaches to detect the formation of these immuno-complexes have been strongly improved over the last years. cGMP detection assays have been developed from laborious hands-on RIAs to automatable homogenous assay formats capable

of processing a large number of samples with high sensitivity. This development also resulted in the stepwise replacement of classical radioactive enzyme activity assays.

The present article reviews the historical development of both major lines and some sideways of the biochemical detection of cGMP from the pioneering work of the first publications to the developments of high throughput screening applications, which have recently become commercially available.

2 Biochemical Detection of cGMP

2.1 Detection of cGMP from Enzymatic Guanylate Cyclase Assays

Guanylate cyclase assays with purified enzyme, cell lysates, or tissue homogenates are indispensable for the investigation of the mechanisms involved in the activation/deactivation of the enzyme, the characterization of its kinetic parameters, and for identifying compounds that modulate the catalytic activity. The general approach is based on the detection of labelled cGMP formed from labelled GTP via the enzymatic activity of GC. Historically, GTP is labelled radioactively although PDE assays have been published using fluorescent derivatives of cGMP (Ohba et al. 2001). The lynchpin of radioactive GC assays is the separation of radioactive cGMP from its precursor GTP. To achieve this aim, various methods have been established since the late 1960s and most of them are not longer applied, with the exception of radioactive sGC assays, which are based on column chromatography.

2.1.1 Radioactive Guanylate Cyclase Assays

cGMP Detection by Thin Layer Chromatography (TLC)

One of the first published methods to quantify cGMP generated in enzymatic assays using cell lysates was based on the conversion of radioactive GTP to radioactive cGMP and its subsequent purification by thin layer chromatography (TLC) (Schultz et al. 1969; White and Aurbach 1969; Bohme 1970; Fleischman 1982). ^{14}C-labelled GTP was converted to ^{14}C-cGMP by sGC-containing supernatants of crude cell lysates, the reaction mixture was spotted on PEI cellulose and separated by TLC. To validate that the observed TLC-signal was due to radioactive cGMP, the corresponding spot was eluted and half of the sample was subjected to a combination of phosphodiesterase (PDE) and alkaline phosphatase (AP) treatment to specifically degrade generated cGMP to radioactive guanosine. Finally, anion-exchange resins adsorbed remaining ^{14}C-cGMP of both samples and the difference of radioactivity measured in the supernatant (due to the radioactive guanosine) of both samples reflected the amount of generated cGMP. Although TLC is able to separate different

nucleotides in parallel, this approach is nowadays only of historical interest due to its limitations with respect to sample throughput and sensitivity. Nevertheless, it allowed the quantification of sGC activity in cellular lysates and anticipated various ideas, which have been incorporated in later approaches for the biochemical detection of cGMP.

cGMP Detection by Column Chromatography

The technical limitations, complex procedure, and the low throughput of the TLC-based purification of radioactive cGMP resulted in the development of various column-based approaches to separate radioactively labelled cGMP from GTP (Murad et al. 1971; Schultz et al. 1973; Krishnan and Krishna 1976; Ward and Brenner 1977; Karczewski and Krause 1978; Gerzer et al. 1981). These techniques are usually applied as part of activity assays with purified GC or enzyme containing cell lysates in which cyclase catalytic activity is monitored via the formation of radioactive cGMP. Although these assays have been established more than two decades ago, they are still routinely applied in many laboratories (Friebe and Koesling 1998; Koglin et al. 2002; Martin et al. 2003; Schmidt et al. 2004; Schindler et al. 2006) due to their high sensitivity, the direct measuring of cGMP, and the possibility to use crude GC-containing cell or tissue lysates as enzyme source. Various articles have been published over the years optimizing the protocol of cGMP purification from cell free assays and cell or tissue lysates. These methods are in general based on the combination of at least two of the following four methods in alternating order: Chromatography of samples on positively charged ion-exchange (IEX) (Murad et al. 1971; Nakazawa and Sano 1974; Schultz et al. 1974; Krishnan and Krishna 1976; Ward and Brenner 1977) or aluminium oxide columns (White and Zenser 1971; Nakazawa and Sano 1974; Krishnan and Krishna 1976; Ward and Brenner 1977; Karczewski and Krause 1978; Gerzer et al. 1981) to retain GTP/GDP and ATP/ADP; separation on negatively charged IEX columns to retain cGMP (Krishna et al. 1968; Schultz et al. 1973; Schultz et al. 1974; Durham 1976); and co-precipitation of adenosine and guanosine mono-, di-, and triphosphates with $ZnCO_3$ which has only slight impact on cyclic nucleotides (Krishna et al. 1968; Schultz et al. 1973; Gerzer et al. 1981). The recovery of cGMP (usually α^{32}P-cGMP) is generally monitored by the addition of trace amounts of ^{3}H-cGMP to the purification procedure.

Still many of the present publications are based on a combination of $ZnCO_3$ precipitation followed by aluminium oxide column chromatography resulting in a cGMP recovery of 65–75% (Gerzer et al. 1981; Schultz and Bohme 1984). As column chromatography is able to directly quantify the increase of cGMP via its radioactivity, there is no need for any enzymatic or fluorescent detection system that could be vulnerable to interfering compounds or colour quenching. These properties in combination with the capability to process up to 96 samples in parallel are making this approach a robust, sensitive, and cheap alternative for many applications especially in the case where crude tissue lysates are used as a source for the enzyme.

2.2 Detection of cGMP in Biological Samples

In addition to the direct determination of the catalytic activity of GCs, there has been and still is a need to determine lowest cGMP levels in biological fluids and tissues e.g., to track physiological pathways affecting GC activity. During the last 40 years, various assays have been developed to achieve this aim thereby pushing the boundaries of sensitivity and sample throughput to the limit of what was technically possible at the respective time. As the commercial available assay kits have become increasingly powerful and sensitive, they are meanwhile also routinely used as read-out for GC catalytic activity, replacing the established radioactive assays described above.

2.2.1 Direct Detection of cGMP

cGMP Detection by High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a well-established approach for the detection of substrates and products of enzymatic reactions in purified samples or complex lysates. As such, HPLC has been used to determine e.g., the enzymatic activity of calcineurin (Enz et al. 1994), dihydropyrimidine dehydrogenase (Lu et al. 1992), phosphodiesterase (Alajoutsijarvi and Nissinen 1987), as well as retinal guanylate cyclase (Koch and Stryer 1988). The major advantage of HPLC for the characterization of enzymatic reactions is its ability to detect unlabelled substrate and corresponding reaction products in parallel. This avoids the need of using labelled substrates such as radioactive of tagged derivatives, which might require special facilities or could influence the enzyme substrate interaction. HPLC-based methods for the detection of GC activity have been published and showed a good correlation with established assays such as RIA (Koch and Stryer 1988; Pietta et al. 1997). Pietta and co-workers reported an optimized HPLC-based protocol for the determination of the enzymatic activity of sGC using a standard C_{18} column and UV-detection at 254 nm. By avoiding a gradient elution profile, the duration of a single HPLC run was reduced to less than 15 min (Pietta et al. 1997). The detection limit of this approach was reported to be 10 pmole cGMP per HPLC run, underlining that the sensitivity of standard HPLC is much lower than commercially available RIAs. One approach to increase the sensitivity of HPLC-based detection of cGMP takes advantage of the fluorogenic reagent (3,4-dimethoxyphenyl) glyoxal (DMPG), to generate fluorescent derivatives of guanosine-containing compounds such as GTP, GDP, or cGMP (Ohba et al. 2001; Soda et al. 2001). The generated fluorescent derivative of cGMP (excitation: 400 nm, emission: 510 nm) was detectable to an amount close to 10 fmol, increasing the detection limit to a comparable level than the antibody-based approaches described below. However, a gradient elution profile was applied resulting in the need for column re-equilibration between two sample injections thus increasing the duration of each run (Soda et al. 2001). A further approach that has been published combined HPLC with mass spectrometry (MS). The

combination of both methods allowed the detection of femtomolar amounts of different nucleotides (e.g. cAMP, cGMP, AMP, and GMP) in parallel (Lorenzetti et al. 2007).

Taken together, HPLC is technically able to detect cGMP in biological samples with high sensitivity (see Table 1). It is the only method that allows the *direct* quantification of cGMP without any need of pre-purification of samples, enzymatic detection systems, labelled cGMP derivatives, radioactivity, or sophisticated data acquisition systems. HPLC offers sensitive quantification of cGMP at affordable costs, if the required hardware (HPLC/MS) is already at hand and established. Although HPLC-based enzyme assays have been shown to be automatable (Pazhanisamy et al. 1995), the major drawback of this approach is the slow serial processing of samples, which limits the use of this versatile method.

2.2.2 Enzymatic Detection of cGMP

cGMP Detection by Enzymatic Cycling

One of the earliest methods for the quantification of cGMP in tissues was the approach of enzymatic cycling described by Hardman and co-workers (Hardman et al. 1966). Enzymatic cycling allowed the direct and sensitive detection of tissue and urine cGMP levels down to femtomolar amounts (Hardman et al. 1966; Goldberg et al. 1969; Ishikawa et al. 1969; Goldberg and Haddox 1974). As enzymatic cycling detects cGMP via its conversion to GDP/GTP, samples had to be purified from such pre-existing contaminants by thin layer or column chromatography as described above. Tracer amounts of ^{3}H-cGMP were added to the samples to calculate the recovery rate during purification (Goldberg and Haddox 1974).

The detection system that allowed the quantification of nanomolar amounts of cGMP was the approach of enzymatic cycling, which consists of two enzymatic reactions: The reaction of Succinate-CoA and GTP to Succinyl-CoA and GDP catalyzed by succinate-thiokinase. Subsequently, the formed GDP is re-phosphorylated to GTP by the enzyme pyruvate kinase resulting in the consumption of Phosphoenolpyruvate and, in parallel, the generation of pyruvate. Thus, the GDP/GTP cycling results in the accumulation of pyruvate equivalents up to three orders of magnitude the amount of the incipiently present GDP (Goldberg et al. 1969). In their initial publication, Goldberg and co-workers quantified the accumulated pyruvate via its conversion to lactate and the consumption of the fluorescent co-substrate NADH. The loss of fluorescence was directly correlated with the amount GDP/GTP present in the assay (Goldberg et al. 1969). To further increase the sensitivity of the assay to femtomolar amounts of cGMP, the detection of NADH was replaced by the direct detection of NAD^{+} upon its conversion into the fluorescent derivative hydroxynicotinaldehyde via the addition of sodium hydroxide (Goldberg and Haddox 1974); an approach that has been rediscovered 25 years later by Seya and co-workers (Seya et al. 1999). To use enzymatic cycling for the detection of cGMP, the sample had to be purified from any contaminating GMP, GDP, and GTP

Table 1

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/ Kit/Price	References
Direct	HPLC	≈10 pmol (standard) ≈10 fmol (fluorescent) ≈30 fmol (LC/MS)	Tubes <30 samples	+ direct detection of cGMP + high sensitivity (fluorescent derivatisation) + detection of various nucleotides in parallel (LC/MS) + cheap (if HPLC or LC/MS equipment is available) + non-radioactive assay + no pre-purification of cGMP necessary + detection is not affected by interfering compounds 0 HPLC is automatable to some extend – slow sample processing and analysis – determination of kinetic parameters not possible – low sample throughput	–	Pietta et al. 1997; Soda et al. 2001; Lorenzetti et al. 2007
Enzymatic	Enzymatic cycling	≈100 pmol (standard) ≈5–100 fmol (fluorescent)	Tubes <30 samples	+ very high sensitivity (fluorescent detection) + high specificity for cGMP + cheap assay + non-radioactive assay – labour-intensive pre-purification of cGMP – possibility of nucleotide-contaminations of enzymes – not automatable – cycling reaction needs to be carefully optimized – compounds might interfere with cycling reaction – low sample throughput – determination of kinetic parameters not possible	–	Hardman et al. 1966; Goldberg et al. 1969, 1974; Seya et al. 1999

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/Kit/Price	References
	GDP-formation and quantification	≈100 fmol	Tubes <30 samples	+ high specificity for cGMP + cheap assay 0 good sensitivity – radioactive assay: demanding special training/facilities – limited half-life of ^{32}P-ATP – labour-intensive pre-purification of cGMP – internal ^{3}H-cGMP standard needed to estimate cGMP recovery – radioactive waste – possibility of nucleotide-contaminations of enzymes – not automatable – compounds might interfere with enzymatic reactions – low sample throughput – determination of kinetic parameters not possible	–	Schultz et al. 1973
	Luciferase bioluminescence	≈10 pmol (384 plate)	96/384/1536 plates >10,000 samples	+ automatable assay format + very high sample throughput – expensive – very low sensitivity for cGMP – designed to detect PDE-induced cGMP degradation – not useful to detect cGMP in biological samples	*Promega PDE-Glo* (V1361, 1,000 test points) (V1362, 10,000 test points) *Costs: 0.35 €/test point*	–

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/Kit/Price	References
Competition-binding	Receptor binding displacement	≈0.5–1 pmol	Tubes <30 samples	+ cheap assay 0 average specificity for cGMP – low sensitivity – radioactive assay: demanding special training/facilities – undefined protein mixture containing cGMP-binding protein – variations in quality of cGMP-binding protein preparations – internal ^{3}H-cGMP standard needed to estimate cGMP recovery – labour-intensive pre-purification of cGMP – not automatable – low sample throughput – determination of kinetic parameters not possible	–	Murad et al. 1971; Gilman and Murad 1974
	RIA	≈100 fmol (standard) ≈3–10 fmol (acetylated)	Tubes <30 samples	+ very high sensitivity (acetylated samples) + very high specificity for cGMP + no need for cGMP pre-purification from other nucleotides 0 relatively expensive kit – radioactive assay: demanding special training/facilities – limited half-life of ^{125}I-cGMP – gamma scintillation counter required – laborious processing of samples prior to assay – not automatable – low sample throughput – determination of kinetic parameters not possible	*GE Healthcare ^{3}H Biotrak cGMP RIA* (TRK500, 150 tubes) *Biomedical Technologies cGMP RIA* (BT-340, 200 tubes) *Izotop ^{125}I cGMP RIA* (RK-525, 100 tubes) *Immunotech cGMP RIA* (IM1118, 100 tubes) *IBL Hamburg ^{125}I cGMP RIA* (RE29071, 100 tubes) *Costs: 2.7–5.5 €/test point*	Steiner et al. 1972; Harper and Brooker 1975; Frandsen and Krishna 1976; Goldberg 1977

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/Kit/Price	References
	SPA	≈200 fmol (standard) ≈4 fmol (acetylated)	Tubes 96 well plates <1,000 samples	+ very high sensitivity (acetylated samples) + very high specificity for cGMP + no need to pre-purify cGMP from other nucleotides + homogenous assay format + automatable + determination of kinetic parameters possible 0 standard luminescence counter needed 0 medium sample throughput − expensive kit − laborious processing of samples prior to assay − radioactive assay: demanding special training/facilities − limited half-life of ^{125}I-cGMP	*GE Healthcare ^{125}I Biotrak SPA* (RPA 540, 100 tubes) *GE Healthcare ^{125}I Biotrak SPA* (RPA 557, 5 × 96plates) *Costs: discontinued*	Hart and Greenwald 1979; Udenfried et al. 1985
	ELISA	≈100–500 fmol (standard) ≈2–5 fmol (acetylated)	96 well plates <1,000 samples	+ very high sensitivity (acetylated samples) + very high specificity for cGMP + no need for cGMP pre-purification from other nucleotides + non-radioactive assay + standard 96 well filter-based plate reader is sufficient 0 medium sample throughput − expensive kit − limited half-life of kits − not automatable − determination of kinetic parameters not possible	*GE Healthcare cGMP direct Biotrak* (RPN 226, 96 plate) *Cayman chemicals cGMP EIA* (581021, 96 plate) *Cambridge Bioscience cGMP EIA* (STA-506, 96 plate) *Assay design cGMP EIA* (900–014, 96 plate) *Biomedical Technologies cGMP EIA* (BT-740, 2 × 96 plates) *IBL Hamburg cGMP ELISA* (CM581021, 8 × 12plate) *Costs: 2.3–5.8 €/test point*	Yamamotho et al. 1982; Pradelles and Grassi 1989; Tsugawa et al. 1991; Horton et al. 1992

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/Kit/Price	References
	LOCI/ AlphaScreen	≈10 fmol (384 well plate)	96/384/1536 plates >10,000 samples	+ very high sensitivity + very high specificity for cGMP + no need for cGMP purification from other nucleotides + automatable + homogenous assay format + very high sample throughput + non-radioactive assay + direct measurement of cGMP in cell lysates + long excitation wavelength reduces background fluorescence 0 relative expensive kit 0 coloured compounds might quench signal 0 reagents are sensitive to ambient light − plate reader needs to be able to excite samples at 680 nm − compounds interacting with reactive oxygen species (anti- oxidants, metal ions) might be able to interfere with read-out	*Perkin Elmer cGMP AlphaScreen* (6760308M, 10,000 test points) (6760308R, 50,000 test points) + *additional Protein A kit* (6760617C, 500 test points) (6760617M, 10,000 test points) (6760617R, 50,000 test points) *Costs: 0.3–2.4 €/test point*	Ullman et al. 1994, 1996
	EFC	≈10 fmol (384 well plate)	96/384/1536/3456 plates >10,000 samples	+ very high sensitivity + very high specificity for cGMP + no need for cGMP purification from other nucleotides + works directly with cell lysates + automatable + homogenous assay format + very high sample throughput + non-radioactive assay + only assay that offers gain-of-signal read-out 0 standard luminescence readers are sufficient (96/384 plates) 0 relative expensive kit − compounds might interfere with enzymatic read-out	*GE Healthcare cGMP HitHunter* (90-0039-01, 100 test points) *discontinued* (90-0039-02, 800 test points) (90-0039-03, 10,000 test points) (90-0039-04, 40,000 test points) *Costs: 0.3–0.8 €/test point*	Kumar et al. 2007 (cAMP); Weber et al. 2002, 2007 (cAMP)

(continued)

Table 1 (continued)

Approach of cGMP detection	Method	Detection limit	Assay format / Sample throughput	Pros and cons	Company/Kit/Price	References
	HTRF	≈40 fmol (384 well plate)	96/384/1536 plates >10,000 samples	+ high sensitivity + very high specificity for cGMP + no need for cGMP purification from other nucleotides + works directly with cell lysates + automatable + homogenous assay format + very high sample throughput + non-radioactive assay – expensive kit – more sophisticated requirements for plate reader (detection of signal at 620 nm and 665 nm, delay between excitation and detection)	*Cisbio HTRF cGMP kit* (62GM2PEB, 1,000 test points) (62GM2PEC, 20,000 test points) *Costs: 0.2–0.6 €/test point*	Mathies 1999; Bazin et al. 2001 (review)
In situ	Immuno-fluorescence	–	Slides <30 samples	+ localisation of cGMP in tissue slides + high specificity for cGMP + acetylation of cGMP in slides has been reported + non-radioactive + cheap – low sensitivity – not suitable for cGMP quantification – labour-intensive preparation of tissues/cells – low sample throughput	–	Chan-Palay and Palay 1979; de Vente et al. 1996

(Goldberg et al. 1969). cGMP was then incubated with phosphodiesterase (PDE) to generate GMP, which was subsequently phosphorylated to GDP via an enzymatic reaction catalyzed by ATP–GMP phosphotransferase under consumption of ATP. The formed GDP was then used to initiate the above-described GDP/GTP cycling reaction allowing the determination of the initial cGMP concentration.

The detection of cGMP by enzymatic cycling is a powerful and non-radioactive approach that offers a detection limit close to 5–10 fmol cGMP per sample at a reasonable price. However, as the cycling reaction is triggered by GDP/GTP, all enzymes and compounds have to be free from any putative contaminations with these molecules to obtain the necessary signal to noise ratio. For the same reason, the cGMP of the samples has to be separated from other guanosine nucleotides. This laborious pre-purification is one of the major drawbacks of the enzymatic cycling approach and reduces dramatically the sample throughput of this method. Moreover, the loss of cGMP during its purification needs to be estimated, what has been usually achieved by the addition of tracer amounts of radioactive cGMP foiling, a least partially the advantage of enzymatic cycling as a non-radioactive approach. In addition, the cycling system needs to be fine tuned to detect cGMP at the highest possible sensitivity. This balance can also be susceptible for interference by chemicals making the enzymatic cycling approach less robust. Nevertheless, once these considerations are taken into account, enzymatic cycling offers a cheap alternative to commercially available antibody-based systems, especially as the direct quantification of NAD^+ as described by Seya and co-workers offers a gain-of-signal read-out in contrast to most other assay formats.

cGMP Detection by Enzymatic Formation of Radioactive GDP

A different enzymatic approach allowing the detection of femtomolar amounts of tissue cGMP was published by Schultz and co-workers (Schultz et al. 1973). Similar to the enzymatic cycling approach published by Hardman and co-workers, the first step for the detection of cGMP relied on its PDE-mediated conversion to GMP. In turn, the generated GMP was used as substrate for subsequent enzymatic reactions, allowing its quantification. In contrast to the non-radioactive approach of enzymatic cycling, the present method is based on radioactive substrates to quantify the generated GMP.

To allow the quantification of cGMP via its conversion to GMP, the sample had to be purified from any cellular GMP to ensure a high signal to noise ratio and to minimize any possible inference of cellular components with subsequent enzymatic reactions involved in the quantification of GMP. Purification of cellular cGMP was achieved by ethanol extraction from homogenized tissues followed by $ZnCO_3$ co-precipitation. Whilst most of the 5′ nucleotides co-precipitated with the formed $ZnCO_3$, cyclic nucleotides such as cAMP and cGMP were virtually not affected and remained in solution. Subsequently, column chromatography was used to purify the sample from residual zinc ions and to separate extracted cGMP from cAMP. The addition of tracer amounts of titrated cGMP to the homogenized sample allowed the

estimation of the overall cGMP recovery, which was calculated to be 40–50%. To quantify the purified cGMP, the sample was subjected to a radioactive enzymatic detection system.

The initial step of this enzymatic detection is comparable to the enzymatic cycling approach. The extracted cGMP was converted to GMP via the addition of PDE. Following heat inactivation of PDE, the generated GMP was phosphorylated by GMP kinase in the presence of the co-substrate γ^{32}P-ATP, resulting in the formation of β^{32}P-GDP and non-radioactive ADP. Remaining radioactive γ^{32}P-ATP was converted to ADP and radioactive inorganic phosphate by adding myosin to the assay mixture. Subsequently, the radioactive phosphate was precipitated by the addition of a mixture consisting of perchloric acid, ammonium molybdate, and triethylamine. The radioactivity found in the supernatant was basically due to the formed β^{32}P-GDP and reflected the amount of the initial cGMP.

This protocol allowed the sensitive quantification of femtomolar amounts of cGMP in biological samples such as cell and tissue homogenates. Although the described method to quantify cGMP via the enzymatic formation of β^{32}P-GDP showed a high sensitivity, it suffered from the same drawbacks than the enzymatic cycling approach described above. The purity of the sample's cGMP from GMP and other cellular contaminants, which might have an impact on the enzymatic detection system, is a prerequisite for the present method and demands a time consuming and labour-intensive purification of the samples. This sample processing obviated any automation of the protocol and resulted in a low sample throughput. In addition, the limited half-life of the radioactive substrate γ^{32}P-ATP and the problematic handling and disposal of radioactive waste would discriminate the present method against the enzymatic cycling approach in its improved version. Despite these considerations, the assay published by Schultz and co-workers offered a cheap option and has been used in the following years for the quantification of cGMP in various tissues (Rabinovitch et al. 1980; Asakawa et al. 1978; Hadden et al. 1976).

cGMP Detection by Luciferase Bioluminescence

Recently, Promega has commercialized a bioluminescent approach for the HTS detection of cellular cAMP, cAMP-Glo. The assay is based on the addition of inactive tetrameric protein kinase A (PKA), PKA substrate, and a luciferase-system to cellular lysates. As the cellular cAMP activates PKA, which in turn consumes ATP to phosphorylate the added substrate, the amount of cellular cAMP is translated into PKA-mediated ATP consumption. As a consequence, the reduced levels of available ATP result in the reduction of luciferase-mediated bioluminescence (Kumar et al. 2007). To protect cellular cAMP levels from degradation, the assay buffer contains also a pan-phosphodiesterase (PDE) inhibitor. The cAMP-Glo assay detects cAMP in cell lysates from low to high femtomolar amounts (Fan and Wood 2007; Kumar et al. 2007).

The same assay approach is commercialized as PDE-Glo to quantify the activity of purified cAMP or cGMP dependent PDEs. The PDE-Glo manual illustrates that not only changes in the cAMP but also in the cGMP concentration can be translated via PKA-activation and subsequent ATP consumption into alterations of the luciferase-induced bioluminescence. However, due to the much lower affinity of PKA for cGMP compared to cAMP, the EC_{50} for the detection of cGMP is increased to approximately 10 pmole. Although this low affinity does not foil the determination of the activity of purified cGMP-specific PDEs (e.g. PDE5), it prevents the use of this bioluminescence approach for the detection of low amounts of cGMP in biological samples. The much higher affinity of PKA for cAMP and the higher cellular concentrations of cAMP compared to cGMP would mask any cGMP-mediated bioluminescence signal foiling the use of this kit for the detection of cGMP in lysates. Although the purification of cGMP from other nucleotides as described for the enzymatic cycling approach would theoretically be able to eliminate cAMP-derived signals, the low affinity of PKA for cGMP would still foil the detection of this molecule with feasible sensitivity. Taken together, it can be concluded that despite PDE-Glo is able to detect PDE-induced cGMP changes in cell free assays, it is non-applicable for the detection of cGMP in biological samples.

2.2.3 Detection of cGMP by Competition-Binding Approaches

Most of the above-mentioned methods for the detection of cGMP in tissue or cell lysates require pre-purification of the samples such as the enzymatic cycling approach (Goldberg et al. 1969; Ishikawa et al. 1969; Goldberg and Haddox 1974) or have a limited sample throughput such as HPLC (Pietta et al. 1997; Soda et al. 2001). In order to overcome these obstacles, competition-binding assays for cGMP have been established. This approach, which forms the basis of most actual assay formats, is based on the competition of cGMP in biological samples and exogenous added labelled cGMP for binding to a common cGMP-binding site. Increasing amounts of unlabelled cGMP from the sample result in displacement of the labelled cGMP from the binding protein and usually (depending on the assay read-out) in a decreasing signal (see Fig. 1). First, competition-binding assays were developed using undefined protein extracts containing cGMP-binding proteins (Murad et al. 1971; Gilman and Murad 1974; Kleine and Kroh 1978); at a later date, the groundbreaking development of anti-cGMP antibodies by Steiner and co-workers (Steiner et al. 1970, 1972b; Richman et al. 1980) allowed the replacement of these protein mixtures by highly specific anti-cGMP antibodies.

Receptor Protein Binding Displacement for cGMP

Before cGMP immuno assays (e.g. RIA, ELISA) became available to a broad range of researchers, different methods based on cyclic nucleotides binding proteins were published (Murad et al. 1971; Gilman and Murad 1974; Kleine and Kroh 1978).

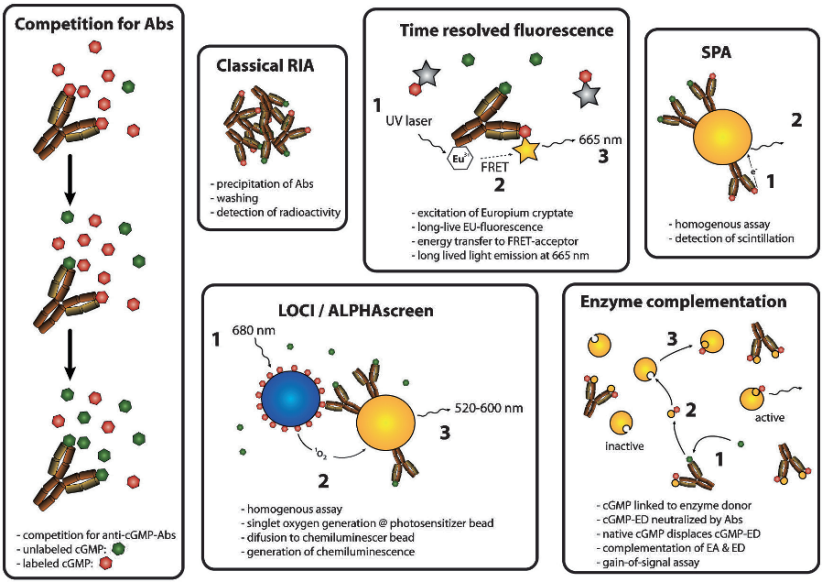

Fig. 1 *Comparison of different competition-based cGMP detection systems*. Virtually all commercially available cGMP detection assays are based on the competition of an unknown amount of unlabeled cGMP from the sample and a defined amount of tracer-labelled cGMP for binding to the anti-cGMP antibodies (Ab). This competition is shown on the left site of Fig. 1 where increasing amounts of unlabeled cGMP from the sample (green) displace tracer-coupled cGMP (red) from the Ab. The subsequent quantification of the displacement differs strongly depending on the assay design. *Classical RIA*: Classical radioimmuno assays (RIA) use radioactively labelled cGMP (usually ^{125}I-cGMP) as tracer and precipitate the Abs. The amount of bound radioactive cGMP can be directly measured in the precipitate. Maximal signal strength is achieved in the absence of sample and increasing amounts of unlabelled cGMP decrease the signal. *SPA*: Scintillation proximity assays (SPA) are comparable to RIAs as they use the same radioactively labelled cGMP. However, in contrast to RIAs the Abs are coupled to the surface of scintillator-containing beads. The radioactive decay of bound ^{125}I-cGMP in the proximity of the bead result in the scintillator-mediated emission of photons whereas electrons emitted from unbound radioactive cGMP are scavenged by the medium. SPA allowed the development of homogenous assay without the need for precipitation and washing steps. *LOCI/ALPHAscreen*: Luminescent oxygen channelling assay (LOCI) is based on the cross linking of two different kinds of microspheres via the interaction of bead-coupled anti-cGMP Abs with biotinylated cGMP (tracer) which in turn binds streptavidin-coupled beads. The proximity of both beads in the presence of Biotin-cGMP allows singlet oxygen molecules generated in one microsphere to react with the acceptor bead resulting in the emission of detectable photons. Singlet oxygen molecules, which are not generated in the proximity of an acceptor bead, are quenched due to the short half-life of this reactive molecule. *HTRF*: Homogenous time resolved fluorescence (HTRF) is based on Foerster resonance energy transfer (FRET) from a FRET-Donor, usually an Europium–Cryptate complex coupled to the Ab, and a FRET acceptor linked to cGMP (tracer). Binding of this fluorescent-cGMP to the antibody allows the detection of a long-lived FRET signal whereas unbound tracer cGMP shows no or only short-lived fluorescence. Maximal signal is achieved in the absence of unlabeled cGMP. *EFC*: Enzyme fragment complementation (EFC) based detection of cGMP is triggered by the spontaneous recombination of two inactive high affinity fragments of β-galactosidase to the active holoenzyme which catalytic activity serves as read-out. The smaller fragment (enzyme donor = ED) is coupled to cGMP (tracer) and neutralised by binding to the anti-cGMP Ab. Addition of unlabeled cGMP from a sample results in the displacement of the ED-cGMP from the Ab and as a result in the formation of active β-galactosidase. EC is the only gain-of-signal assay resulting in higher signal with increasing amounts of unlabeled cGMP

These approaches used undefined protein preparation from various sources containing cAMP- or cGMP-binding proteins such as PKA or PKG to bind the corresponding cyclic nucleotide. With respect to cGMP, Murad and co-workers published a receptor protein displacement assay, which used a protein homogenate from lobster tail muscle as binding protein for cGMP. By using increasing amounts of ^{3}H-cGMP, the authors showed specific binding to proteins from the lobster protein preparation with a K_D value of 2–10 nanomolar depending on the preparation. In the presence of 60 nM ^{3}H-cGMP saturated binding of the radioligand was achieved, thus enabling the detection of picomolar amounts of cGMP. Like for classical receptor binding assay with purified receptors, the addition of unlabelled ligand from purified or non-purified samples resulted in the displacement of radiolabeled cGMP from the lobster protein preparation allowing the quantification of the respective cGMP concentration.

The receptor protein displacement assay allowed the reproducible detection of cGMP in biological samples close to 500 fmol per sample. The major advantage of this method was that it was able to detect cGMP with sufficient sensitivity for many applications for a very reasonable price. In addition, as the assay directly detected the binding of cGMP to its target protein, it was less susceptible for interference by chemicals than enzymatic detection systems. The approach of protein binding displacement was further optimized to allow the parallel detection of cGMP and cAMP in urine samples (Kleine and Kroh 1978). However, the protein mixture used as cGMP receptor showed not only less sensitivity but also less specificity for cGMP compared to immunoassays. Especially, cAMP showed a much higher cross-reactivity to the cGMP-binding protein preparation compared to antibody-based methods. This lack of specificity demanded a labour-intensive pre-purification of cGMP from other nucleotides and resulted in a low sample throughput. Although the protein receptor displacement assay was a reliable and cheap approach, the above-mentioned drawbacks resulted in its replacement by immunoassays such as RIA or ELISA.

cGMP Radio Immuno Assay (RIA)

The development of cGMP-specific antibodies by Steiner and co-workers (Steiner et al. 1972a) enabled the design of a cGMP radio immuno assay (RIA) as described earlier for cAMP (Steiner et al. 1969). As cyclic nucleotides are not immunogenic, a 2′-O-succinyl derivative of cGMP was coupled to keyhole limpet hemocyanin, which was used to immunize rabbits (Steiner et al. 1972a). The generated antibodies showed a high selectivity for cGMP and virtually no cross-reactivity with other nucleotides rendering sample pre-purification unnecessary (Steiner et al. 1972a, b). The published RIA was a classical competition-binding assay comparable to the protein cGMP displacement assay described earlier (Murad et al. 1971) but with much higher sensitivity allowing the detection of tissue cGMP levels down to 100 fmol per sample. This increase in sensitivity was achieved by the higher affinity of the antibody for cGMP compared to cGMP-binding proteins and the use of

^{125}I-cGMP (Steiner et al. 1972a), which has a much higher specific activity than ^{3}H or ^{14}C labelled cGMP. Tissue cGMP displaced the radioactively labelled cGMP from the antibody until equilibrium between association and dissociation of both ligands was reached (Steiner et al. 1972b, Fig. 1). Subsequently, antibody-bound and free ^{125}I-cGMP were separated via precipitation of the anti-cGMP antibody by adding secondary anti-rabbit antibodies, ammonium sulphate (Steiner et al. 1972b), or ice-cold ethanol (Frandsen and Krishna 1976). Alternatively, unbound cGMP was adsorbed by charcoal (Goldberg 1977). The initial cGMP RIA as published by Steiner and co-workers (Steiner et al. 1972b) was further optimized to higher sensitivity by acetylation or succinylation of the sample's cGMP (Harper and Brooker 1975; Frandsen and Krishna 1976). By generating these derivatives, which reassembled more closely the cGMP derivative that was used for immunization (Steiner et al. 1972b; Harper and Brooker 1975; Frandsen and Krishna 1976; Brooker et al. 1979), the detection limit of this approach was reduced to 3–10 fmol per sample (Harper and Brooker 1975; Frandsen and Krishna 1976). This extremely high sensitivity combined with the lack of virtually any cross-reaction with other nucleotides made this assay extremely useful for the detection of tissue cGMP levels and formed the basis for actual commercial cGMP RIA kits.

Despite the unmatched sensitivity and specificity for cGMP, the classical RIA has several drawbacks that promoted the development of more sophisticated detection systems. The classical RIA relies on a time consuming extraction of cGMP from tissue or also cell homogenates. Usually this includes lyophilization and subsequent resuspension of the samples in assay buffer. As this process is not automatable, it hampers the analysis of large numbers of samples. Furthermore, the general RIA protocol requires incubation over night, and antibody-bound cGMP needs to be separated from free ligand by several precipitation and washing steps slowing the analysis of samples further down. Another drawback lies in the use of radioactive cGMP as tracer. Most commercially available RIAs use ^{125}I-labelled cGMP that offers, on the one hand, a high specific activity thereby increasing the sensitivity of the assay; on the other hand, it reduces the shelf life of the kit due to the short half-life of the isotope. In addition, the use of radioactive tracers demands special facilities and waste disposal procedures. Despite these obstacles and the high costs of commercially available cGMP RIAs, the high sensitivity and specificity are making this assay a well-established option to quantify cGMP in a manageable number of samples.

cGMP Scintillation Proximity Assays (SPA)

The development of RIAs enabled researchers to detect cGMP from biological samples or enzymatic assays with an unmatched sensitivity at that time. Nevertheless, as described above, classical RIAs suffer from several drawbacks mainly the labour-intensive sample processing. Moreover, RIAs rely on the equilibrium of binding of the ligand to the antibody; as such this approach does not allow the determination of association and dissociation kinetics. Although this doesn't affect the detection

of cGMP in biological samples, the ability to monitor binding kinetics of ligands to their corresponding target proteins has been desirable for various applications e.g. drug development. The scintillation proximity assay (SPA) resolved most of these obstacles as it allowed the development of homogenous assay formats without any need for precipitation or washing steps.

The term scintillation proximity assay for a special class of assay technique was coined by Hart and Greenwald (Hart and Greenwald 1979a, b). In these initial publications, the authors determined the interaction of antibody and antigen by using two different types of antigen-coated microspheres of which one was ^{3}H-labelled, whereas the other contained a scintillator. As the β-particles emitted from of the ^{3}H-labelled beads had an average energy of 6 keV, they were readily absorbed in aqueous buffers within very short distances of approximately 4 μm. However, once both types of microbeads were cross linked via the addition of appropriate antibodies, the β-particles were emitted in the direct proximity of the scintillator resulting in the emission of a photon, which could be detected by a standard scintillation counter (Hart and Greenwald 1979a, b). Although the described application was designed for the quantification of antibody titres, the authors already pointed out that coating of the microspheres with appropriate receptors such as antibodies could convert SPA into an extremely versatile technique (Hart and Greenwald 1979b).

In the following years, the SPA approach was optimized to increase its sensitivity and versatility. A major development was the use of antibody coated scintillator-containing microspheres in combination with radioactive ligands (Udenfriend et al. 1985, 1987; Bosworth and Towers 1989). By replacing the ^{3}H labelled microspheres of the original assay design (Hart and Greenwald 1979a) with directly radioactively labelled ligands, the binding reaction was turned from ternary into second order reducing the time needed for the formation of antibody–antigen complexes (Udenfriend et al. 1985). A further increase in sensitivity was achieved by using ^{125}I as radioactive label. The higher specific activity of the decay of ^{125}I to ^{125}Te combined the higher average energy (35 keV) and operating distance in (35 μm) of the emitted Auger electrons resulted in an increased emission of photons from the scintillator microbeads (Udenfriend et al. 1985, 1987). An additional advantage of the use of ^{125}I is that ligands can be labelled by a broad range of well-established and commercially available methods and kits for iodination, whereas the synthesis of ^{3}H-labelled ligands is usually more complex. Although it is possible to establish SPAs with other isotopes commonly used in life science applications (e.g. ^{14}C, ^{35}S), only few publications describe such an approach. The β particles emitted by these isotopes are too energetic resulting in increased path length in aqueous buffer and thereby in higher background signals due to unspecific activation of scintillator beads (Udenfriend et al. 1985; Carpenter et al. 2002). The SPA beads are generally available in two types: inorganic Yttrium silicate (YSi) or organic Polyvinyltoluene (PVT) containing diphenylanthracine as scintillator. Although YSi is one of the most efficient solid scintillators with an average counting efficiency of 60% compared to liquid-scintillation counting, the high density of YSi beads of $\approx$4 g cm^{-3} results in faster settling compared to the lower density PVT beads (1.05 g cm^{-3}) (Carpenter et al. 2002). The SPA approach in general offers a higher versatility as it allows to

determine association and dissociation kinetics of radioactive ligands to their target protein. However, although this feature might be highly desirable for drug discovery applications, it is not essentially needed for the quantification of cGMP in biological samples.

The commercially available cGMP SPA kits are based on PVT microspheres coated with anti-rabbit antibodies and primary rabbit anti-cGMP antibodies raised against 2′-O-succinyl derivative of cGMP as described by Steiner and co-workers (Steiner et al. 1972a). cGMP is quantified by displacement of the radioactive cGMP tracer from the antibody resulting in reduced scintillation rates (Fig. 1). Due to its advantages compared to tritium labelled tracers, the available SPA kits are using ^{125}I-cGMP as ligand. However, as mentioned above for RIA, the use of this isotope as tracer reduces the shelf life of the assay kit. Beside the use of radioactive tracers, SPA has similar drawbacks than RIA, namely the labour-intensive sample processing prior to cGMP determination. Although both methods have similar sensitivity and specificity, SPA offers several advantages. As a homogenous assay format, SPA has no need for precipitation and washing steps strongly facilitating the assay and allowing its automation. The use of 96 well plates and scintillator beads emitting photons in the visible spectrum (e.g. 420 nm) permit signal detection with a standard luminescence counter albeit the possibility of signal quenching due to coloured compounds has to be taken into account. In summary, compared to RIA, the SPA approach offers higher versatility and sample throughput with matching sensitivity and specificity at comparable high costs.

cGMP Enzyme Linked Immunosorbent Assay (ELISA)

The development of specific antibodies to succinylated cGMP–protein conjugates (Steiner et al. 1972a, b; Richman et al. 1980) allowed the development of various cGMP RIA and SPA approaches for the detection of femtomolar amounts of cGMP (see above). To maximize the sensitivity, most assay kits used ^{125}I-cGMP as tracer as its specific activity is much higher compared to ^{3}H- or ^{14}C-cGMP. The high specific activity of ^{125}I reflects its relative short half-life of 59.4 days demanding newly synthesized ^{125}I-cGMP every few weeks. In addition, there are further drawbacks of radioactive assays in general: Handling with radioactive compounds demand special needs for storage, waste disposal, and lab hardware as well as staff training to work with radioactive samples. These obstacles led to the development of non-radioactive enzyme-linked immunosorbent assays to quantify cGMP levels with comparable sensitivity as RIA.

Various authors published non-radioactive ELISA approaches for the detection of cGMP. Virtually all methods are based on polyclonal rabbit anti-cGMP antibodies raised against succinylated cGMP coupled to bovine, human, or rabbit serum albumin (Yamamoto et al. 1982; Horton et al. 1992; Wellard et al. 2004) or keyhole limpet hemocyanin (Pradelles et al. 1989). Tsugawa and co-workers published a cGMP-ELISA based on a monoclonal antibody directed against succinylated cGMP coupled to human serum albumin (Tsugawa et al. 1991). Due to the small molecular

weight of cGMP, it is hardly possible to generate antibodies against different epitopes, which don't interfere with each others binding to the molecule. Since the independent binding of two antibodies is a prerequisite for direct sandwich ELISAs, all published enzyme-linked approaches are designed as competitive ELISA using only one specific anti-cGMP antibody. Different assay designs have been implemented. Some approaches used the initial immunogen (cGMP coupled to rabbit or human serum albumin) to coat microtitre plates (Tsugawa et al. 1991; Wellard et al. 2004). Upon reaching equilibrium of binding between plate-bound HSA-cGMP and free cGMP to the anti-cGMP antibody, the sample's cGMP concentration was reflected by the amount of plate-bound antibody. This primary antibody was subsequently quantified via commercial available secondary antibodies coupled to detection enzymes such as alkaline phosphatase (AP) or horse radish peroxidase (HRP). Other publications established the coating of surfaces such as microtitre plates (Pradelles and Grassi 1989; Horton et al. 1992) or polystyrene particles (Yamamoto et al. 1982) with secondary anti-rabbit antibodies to bind the primary anti-cGMP antibody. Subsequently, the sample's cGMP competes for binding to the primary antibody with cGMP-coupled detection enzymes such as acetylcholine esterase (Pradelles et al. 1989), HRP (Horton et al. 1992), or β-galactosidase (Yamamoto et al. 1982), allowing the quantification of cGMP via a colorimetric enzyme reaction. However, this assay design demands special cGMP tracer such as HRP–cGMP, which in turn determines the enzymatic detection reaction.

The approach to coat microtitre plates with the initial immunogen like HSA–cGMP offers a broader versatility as standard commercially available secondary anti-rabbit antibodies with different coupled detection systems can be used to detect the amount of bound primary anti-cGMP antibody. All published as well as commercially available assays are, independently from their design, able to detect low femtomolar amounts of cGMP in biological samples offering the same range of sensitivity as RIA or SPA. In contrast to these assays, ELISA, as an entirely non-radioactive approach, does not require any special facilities or waste disposal. Samples including crude cell lysates can be directly measured without any time consuming extraction and lyophilization procedure increasing the feasibility and sample throughput of this assay format compared to available RIA and SPA kits. Although ELISA demands various washing steps (in contrast to the homogenous assay formats such as SPA), it can be automated to some extent allowing the processing of a fair number of samples in manageable time. Moreover, the extensive wash steps impede any interference of coloured chemicals or sample components with the subsequent enzymatic detection. Depending of the detection system and the chromogenic substrate, data acquisition can be accomplished on standard filter-equipped 96-well plate readers. Although no radioactive tracers are used, the shelf life of some commercially available cGMP-ELISA is in the same range as described for RIA and SPA. Nevertheless, shelf life as well as price of cGMP-ELISAs can vary strongly between different manufacturers (see Table 1). In general, the price per kit is in the same range as for RIA or SPA. Taken together, the matching sensitivity and specificity for cGMP compared to RIA/SPA, the use of non-radioactive reagents

and standard filter-based plate readers, and the possibility to process a fair number of samples make cGMP-ELISAs a good choice for many labs.

Detection of cGMP via Luminescent Oxygen Channelling Assay (LOCI)

The luminescent oxygen channelling assay (LOCI) or ALPHAscreen was first described by Ullman and co-workers (Ullman et al. 1994). Generally, the assay is based on the same principles as the incipiently published scintillation proximity assay (Hart and Greenwald 1979a). Both approaches, SPA and LOCI, crosslink via an antigen–antibody interaction, two different kind of microspheres, whose proximity result in the emission of detectable photons (Hart and Greenwald 1979a; Ullman et al. 1996). However, in contrast to SPAs in which the close proximity of radioisotope and scintillator is the basis for the emission of photons, LOCI represents an entirely non-radioactive approach.

The LOCI technique is based on two species of coated beads of which one has dissolved in it a photosensitizer whereas the other contains the chemiluminescer. The photosensitizer beads are loaded with the dye Phthalocyanine that shows a strong absorption at wavelength of 680 nm allowing the use of commercially available 680 nm-solid-state lasers for excitation. Once activated, the dye generates a highly instable singlet oxygen molecule, which is able to diffuse approximately 200 nm before falling back into the ground state. The short half-life of the singlet oxygen ensures a low background signal of the LOCI approach, as the $^1O_2$ source has to be in very close proximity to the $^1O_2$ acceptor to generate chemiluminescence. The $^1O_2$ accepting particles contain the alkene Thioxene, which rapidly reacts with singlet oxygen molecules resulting in chemiluminescence at 390 nm (Ullman et al. 1996). This energy is instantaneously transferred to fluorophores (e.g. bisphenylethynylanthracene) dissolved in the same microsphere shifting the emission to a wavelength of 520–600 nm (Ullman et al. 1996; Gabriel et al. 2003). This emission (upon excitation at 680 nm) directly correlates with the amount of photosensitizer–chemiluminescer particle pairs and allow therefore the quantification of the antigen–antibody interaction. Both kind of particles are coated with a dextran-based hydrogel that strongly diminished unspecific interactions and prevents due to its hydrophilicity sedimentation of the particles in solution (Ullman et al. 1994, 1996).

With respect to the quantification of cGMP, Perkin Elmer commercializes the LOCI approach as ALPHAscreen. In this assay kit, the photosensitizer beads have bound streptavidin to their surface, whereas the chemiluminescent particles are coated (via protein A) with an anti-cGMP antibody. The ALPHAscreen is designed as a competitive assay: Unlabelled and biotin-tagged cGMP compete for the antibody-binding sites. In the absence of unlabelled cGMP, the biotinylated cGMP results in the maximum crosslink of both beads and therefore in a maximum signal. Increasing amounts of unlabelled cGMP result in the displacement of biotin-cGMP from the antibody-binding site and, as a consequence, in the dissociation of the $^1O_2$-donor–acceptor particle pairs. In the presence of 1 μM unlabelled cGMP, the laser-induced emission of light is virtually abolished.

ALPHAscreen is capable of detecting low femtomolar amounts of cGMP in samples suggesting that this approach offers a comparable sensitivity than RIA, SPA, or ELISA. ALPHAscreen, as a homogenous assay format, can be readily automated and allows the determination of cGMP in cell free and cellular assays in 96, 384, or 1,536 well plates. In contrast to RIA and SPA, cellular cGMP can be directly quantified in crude cell lysates with high sensitivity and specificity, thus allowing the processing of a large number of samples and rendering time consuming extraction procedures unnecessary. ALPHAscreen is not based on enzymatic detection systems making this assay format less vulnerable to compounds, which might have the potency to interfere with enzymatic read-out systems. Nevertheless, as the emitted light is in the visible spectrum the signal strength can be affected by colour quenching chemicals. Also it has to be taken into account that antioxidants or metal ions might be able to scavenge the singlet oxygen released from the photosensitizer beads. The relatively low excitation energy at a wavelength of 680 nm results in reduced background fluorescence; however, it demands a data acquisition system compatible with the required excitation and emission wavelengths. ALPHAscreen in principle is able to continuously monitor cGMP accumulation and/or degradation in assays using purified enzyme preparations or cell lysates. The assay kit is expensive as it has to be combined with an additional Protein A kit (see Table 1). Furthermore, the ALPHAscreen cGMP kit is only available for analysing 10,000 or 50,000 samples, what might be oversized for many laboratories. In contrast, the necessary Protein A kit is already available at a kit size of 500 samples. Nevertheless, the price of the kit is very competitive, if very large numbers of samples have to be screened. Due to its novelty compared to established assay formats, it is hard to estimate the feasibility of ALPHAscreen for the detection of cGMP apart from its use as HTS assay. If appropriate plate readers are present, the sensitivity of the assay and its capability to monitor and quantify cGMP directly in cell lysates are making ALPHAscreen an option compared to RIA, SPA, or ELISA.

Detection of cGMP by Enzyme Fragment Complementation Assays (EFC)

Enzyme fragment complementation (EFC) assays are based on the principle that various enzymes such as β-galactosidase, β-lactamase, luciferase, or dihydrofolatereductase can be genetically split into two independent expressible and catalytic inactive fragments (enzyme donor: ED, enzyme acceptor: EA) which can, however, recombine to form the holoenzyme and thereby regain catalytic activity (Remy and Michnick 1999; Rojo-Niersbach et al. 2000; Ozawa et al. 2001; Wehrman et al. 2002; Weber et al. 2007). EFC has been adapted to fit different assay strategies depending on the kind of interaction that needs to be investigated. In order to track protein–protein interactions, the proteins of interest can be genetically fused to the EA and the ED, respectively. Once the proteins interact the fused EA/ED pair form the holoenzyme allowing to follow the process of protein interactions via the regained catalytic activity. To avoid a possible impact of the enzyme fragments onto

the interaction of the fused proteins, the enzyme fragments have been engineered to show a rather low affinity for each other (Eglen 2002; Golla and Seethala 2002).

The commercially available cGMP detection kit 'HitHunter' from DiscoveRx is based on a slightly modified strategy. In contrast to the low affinity EA/ED pair described above, HitHunter takes advantage of a high affinity ($K_D \approx 1\,nM$) interaction of two engineered fragments of *E. Coli* β-galactosidase (Eglen 2002; Golla and Seethala 2002). The assay is based on the spontaneous recombination of the large catalytically inactive enzyme fragment (enzyme acceptor, EA) and the approximately 4 kDa small enzyme donor. In order to link enzyme complementation to cGMP concentrations, the small enzyme donor is chemically conjugated to cGMP. Although this ED-tagged cGMP is able to trigger the high affinity enzyme complementation with the EA, this process is blocked by the presence of anti-cGMP antibodies in the assay mixture neutralizing the available ED-cGMP (Eglen 2002; Golla and Seethala 2002; Weber et al. 2004). Increasing amounts of native cGMP produced either by guanylate cyclase activity assays or by the addition of cGMP containing samples to the assay mixture, result in a concentration dependent displacement of ED-cGMP from the anti-cGMP antibodies. Free ED-cGMP immediately binds to the EA fragment forming enzymatically active β-galactosidase, which in term catalyses the conversion of a chemiluminescent substrate, such as dioxetane (Weber et al. 2004). Thereby, the HitHunter EFC approach directly links cGMP concentration to the emission of chemiluminescence which can be quantified by standard scintillation readers (Weber et al. 2004). HitHunter is capable of detecting cGMP in biological samples at femtomolar amounts matching the sensitivity of the above-described ALPHAscreen. The manufacturer's protocol recommends the use of HitHunter with 96 or 384 well plates. However, the comparable HitHunter cAMP detection kit was successfully miniaturized to 1,536 and even 3,456 well plates (Weber et al. 2004, 2007).

The pros and cons of HitHunter reassemble most of the considerations mentioned above for ALPHAscreen. The assay directly detects cGMP in crude cell lysates as well as in cell free assays, dramatically reducing labour-intensive preprocessing of the sample, prior to measuring. HitHunter can be automated thus allowing the processing of a very large number of samples. The use of chemiluminescent substrate for the β-galactosidase read-out system enables the use of standard photomultiplier based data acquisition systems. Theoretically, as the addition of substrate is the last step in the assay protocol, any of the commercially available fluorogenic or chromogenic substrates for β-galactosidase might be used (see http://www.sigmaaldrich.com/catalog/search/TablePage/15845000), although the sensitivity of the assay could be compromised. Nevertheless, the free choice of substrate would allow adapting HitHunter to the detection hardware available in the respective lab, and if used in a 96 well format also chromogenic substrates might offer sufficient sensitivity. The assay kit is sold at a competitive price (see Table 1) and, in contrast to ALPHAscreen, in a kit size that matches the demands of many laboratories (800 test points). As HitHunter uses an enzymatic read-out system, one has to be aware that compounds able to inhibit β-galactosidase might affect the signal strength. A major advantage of the assay is that, in contrast to virtually

all other competition-based methods, the EFC offers a gain-of-signal read-out with increasing amounts of cGMP. Taken together, HitHunter represents a very promising approach for the detection of cGMP not only for HTS but also non-HTS applications, as it offers a non-radioactive assay format able to detect cGMP with high sensitivity and specificity even in crude cell lysates. Despite its capability to be miniaturized to 1,536 or even 3,456 well plates might not be of interest for many applications, the combination of a gain-of-signal read-out with the possibility to adapt the assay to pre-existing hardware by choosing different substrates makes HitHunter a very attractive choice for the detection of cGMP.

Detection of cGMP by Time Resolved Fluorescence (TRF)

Fluorescence techniques for the detection of protein–protein or protein ligand interactions have become increasingly important over the last decade. Various approaches have been developed including dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA), fluorescence polarization (FP), fluorescence resonance energy transfer (FRET), and homogenous time resolved fluorescence (HTRF) (Hemmila and Webb 1997; Trinquet and Mathis 2006). These assays have been published and commercialized for the detection of various ligands including cAMP. Although an adaptation would have been unproblematic, at present only HTRF has been described for the detection of cGMP.

HTRF is based on the radiation-less energy transfer of from a donor- to an acceptor-fluorophore via Foerster resonance energy transfer (FRET). Thereby, the energy from the excited FRET donor (e.g. via UV-light) is partially transferred to the FRET acceptor, which emits light of a lower wavelength. As FRET is very sensitive to the distance of both molecules, this approach can be used to detect the interaction of a labelled ligand (e.g. cGMP) with a labelled receptor (e.g. anti-cGMP antibody). However, one of the major drawbacks in the development of such an approach has been the background fluorescence of complex assay mixtures such as cell lysates or human sera, which resulted in serious limitations of sensitivity (Mathis 1993). Dilution of samples reduced the impact of the background fluorescence but in turn also decreased the sensitivity. As most background fluorescence is very short-lived (≈ 4 ns; Trinquet and Mathis 2006), the use of rare earth chelates such as Europium (Eu), Terbium (Tb), or Samarium (Sm) as FRET donors represented a major progress in the development of HTRF. The extraordinary long fluorescence lifetimes of rare earth chelates of up to $1,000\,\mu$s combined with time resolved fluorescence detection allowed the temporal discrimination between background and label fluorescence (Mathis 1993, 1995, 1999). Later the thermodynamically more stable cryptates were developed as FRET donors (Mathis 1999; Bazin et al. 2001). Eu^{3+}-Cryptates (Eu^{3+}-tris-bipyridine) are stable in aqueous buffers and can be readily linked to biomolecules such as antibodies. The cryptate-molecule complexes the europium ion, shields it from interactions with signal-quenching buffer components, and transfers absorbed energy to the metal ion (Mathis 1995, 1999). In the case that

the excited europium cryptate is in close proximity to the FRET acceptor XL665 (a chemically stabilized derivative of the algae fluorophore allophycocyanin (Trinquet et al. 2001)), the energy can be partially transferred to this fluorophore and is subsequently emitted as a detectable photon. As the emission spectra of XL665 show virtually no overlap with the emission spectra of the europium–cryptate, both fluorophores can be easily discriminated.

The cGMP HTRF assay is designed as competitive immunoassay. An Europium–cryptate complex (FRET donor) is coupled to the anti-cGMP antibody, whereas the FRET acceptor XL665 is chemically conjugated to cGMP. Once interaction takes place, the antibody-conjugated europium–cryptate complex (activated by an UV-laser pulse) can transfer energy to the FRET-acceptor linked to cGMP. Due to the long lifetime of the activated cryptate, the background fluorescence has already died away when energy is transferred to the FRET acceptor, strongly increasing the signal-to-noise ratio. Subsequently, the FRET-activated cGMP–XL665 molecule emits fluorescence at a wavelength of 665 nm. In the absence of unlabelled cGMP, maximum signal can be detected. Increasing amounts of cGMP in the assay mixture displace the XL665–cGMP complex from the europium–cryptate–antibody complex and result in a concentration dependent decrease of the emission at 665 nm. The cGMP HTRF assay has been described to detect femtomolar amounts of cGMP in biological samples.

HTRF is able to detect cGMP in cell free assays as well as in crude cell lysates rendering the processing of samples prior to cGMP detection unnecessary. As homogenous assay, HTRF can be readily automated and miniaturized to 96-, 384-, or 1,536-well plates allowing the screening of very large numbers of samples. The sensitivity of HTRF for cGMP has been reported to be slightly lower than for other competition based assay formats (see Table 1). However, due to the novelty of the HTRF-based cGMP detection kit, no independent papers have been published yet corroborating the reported sensitivity. As a biophysical approach, HTRF is not as vulnerable to potential enzyme inhibitors as EFC-based assays like HitHunter are. Also antioxidants, which could affect the ALPHAscreen signalling, are unlikely to have an impact on HTRF. One of initial problems in the development of HTRF was the UV absorption of media or cell lysates quenching the available energy to excite the Europium–cryptate complex. This absorption-induced lack of energy would have reduced the cGMP–XL656 light emission resulting in false-positive readings. The parallel detection of the FRET-based fluorescence at 665 nm and a reference signal at 620 nm solved this problem. For this reason, data acquisition systems compatible with HTRF have to be capable of detecting both wavelengths in parallel and, in addition, to handle the delayed light emission after UV excitation. These pre-requisites indicate that HTRF requires more sophisticated plate readers compared to other competitive immunoassays, what might be the major drawback of the HTRF-based detection of cGMP. If compatible hardware is at hand, HTRF offers cGMP detection with high sensitivity and sample throughput for a reasonable price.

2.2.4 cGMP Immunofluorescence

The development of cGMP-specific antibodies (Steiner et al. 1969, 1972b) allowed the development of highly sensitive and reliable biochemical cGMP detection assays as described above. In parallel, these antibodies raised the possibility to detect and localize cGMP in tissues or cells by immuno histochemical (IHC) approaches (Bloom et al. 1972; Wedner et al. 1972). The detection of water-soluble molecules of low molecular weight such as cAMP or cGMP by IHC generally suffers from several drawbacks. One problem is due to the different derivatization of cyclic nucleotides to achieve immunogenic cGMP/cAMP–protein conjugates. Whereas specific antibodies were raised against conjugates of the 2′-succinylated derivatives of the corresponding cyclic nucleotides (Steiner et al. 1969, 1972a), the widely used IHC protocols are based on the formaldehyde or paraformaldehyde fixation of tissues, which result in different derivatizations of cGMP compared to the initial immunogen. Early publications describing IHC for the detection of cAMP omitted therefore any fixation step to avoid the formation of less immunogenic derivatives of cellular cAMP (Wedner et al. 1972). As the omission of tissue fixation resulted in the loss of most cellular cyclic nucleotides (Cumming et al. 1980; Ortez 1980), later publications applied a paraformaldehyde (de Vente and Steinbusch 1992) or formaldehyde fixation, which retained approximately 30% of cellular cGMP levels (de Vente et al. 1996). To overcome the problem of cGMP derivatization by formaldehyde, de Vente and co-workers raised specific antibodies against formaldehyde fixed cGMP–protein conjugates (de Vente et al. 1987). Although this approach offered some advantages, the feasibility and specificity of the commercially available and widely used antibodies against succinylated derivatives of cGMP for IHC in formaldehyde- and paraformaldehyde-fixed tissues was shown by different publications and methods (Mehlhorn et al. 2000; Bloch et al. 2001; Korkmaz et al. 2005; Tian and Yang 2006; Werkstrom et al. 2006) such as gas phase acetylation of tissue cAMP, which resulted in the same increase in sensitivity as observed in RIAs (Chan-Palay and Palay 1979; Rosenberg et al. 1979). Nowadays, the most widely applied IHC protocols are based on 3–4% formaldehyde (de Vente et al. 1996; Mehlhorn et al. 2000) or paraformaldehyde-fixed tissues (de Vente and Steinbusch 1992; Bloch et al. 2001; Tian and Yang 2006), permeabilization, and subsequent cGMP detection by commercial available antibodies raised against the succinylated derivatives of cGMP in a dilution range from 1:100 to 1:600. cGMP immunofluorescence is not comparable to any of the other biochemical approaches to detect cGMP, as the goal of IHC is not the precise quantification of cGMP with the highest possible sensitivity but its intracellular localization in tissue slides or cells. Thus cGMP IHC is able to offer unique information about the distribution of cGMP. To some extent, IHC is also able to detect alterations of tissue cGMP concentrations, however, this detection is of a more qualitative rather than quantitative character. cGMP IHC is a well-established method able to give valuable complementary information about the distribution of cGMP in tissues. Nevertheless, this method has to be accompanied by one of the above-mentioned cGMP detection assays if the accurate quantification of cGMP is of interest.

3 Summary

Four decades of cGMP research have left today's scientists in the beneficial situation to choose from a broad variety of established and commercialized methods to quantify cGMP derived from enzymatic assays or extracted from biological samples. All the methods presented here can be successfully used to quantify cGMP; nevertheless, the applied approach should be chosen carefully to meet the respective demands. The perfect cGMP detection assay should be: highly sensitive and specific, non-radioactive, cheap, easy to handle, able to directly detect cGMP in samples without any purification, offer high sample throughput, be robust against any kind of interference, and adaptable to any kind of standard plate reader. None of the described approaches is able to meet all of these requirements; however, it is easily possible to narrow down the choice based on the respective research aims and lab equipment.

The primary goal, the quantification of cGMP with sufficient sensitivity, is fulfilled by all presented assays with an exception of PDE-Glo. This PKA-based luminescent approach has been designed to detect the PDE-induced decrease of artificially high cGMP concentrations, making it unfeasible for the detection of low levels of cGMP in biological samples. Next, it should be taken into account that the use of radioactivity as detection system has some severe drawbacks, namely the half-life of some common isotopes, the disposal of waste, the need for special facilities, staff training, and hardware to quantify radioactive samples. To avoid these problems, and as there are various non-radioactive alternatives of matching or even higher sensitivity available, it might be worth to decide against radioactive approaches such as RIA or SPA. In addition, these assays demand labour-intensive sample purification, drastically reducing the sample throughput.

From this short list, the appropriate approach should be chosen pragmatically based on the respective needs e.g. estimated usage and sample throughput, price, and compatibility with existing data acquisition hardware. For the infrequent cGMP detection of a manageable amount of samples commercially available ELISAs represent a good choice. Although these kits are quite expensive compared to the amount of samples that can be processed, ELISAs offer high sensitivity, are easy to handle, allow the detection of cGMP in cell lysates without pre-purification and are compatible with most 96 well plate readers. If cGMP needs to be detected more frequently, commercial ELISAs can become an expensive choice and other methods might be taken into consideration. In the case that HPLC and or LC/MS hardware is at hand and established, the direct quantification of cGMP can be a good choice at a reasonable price. The sensitivity, especially when combined with MS is very good, and if sample injection and data processing is automated, a fair amount of samples can be analysed. Also, the approach of enzymatic cycling might be considered as cheap and sensitive alternative that can be adapted to many plate readers. However, as it might take some time to establish and optimize the assay conditions, the amount of samples should be high enough to justify the effort. Moreover, it should be considered that samples have to undergo a pre-purification procedure prior to the determination of cGMP levels.

Recently, three assay formats developed for high throughput screenings, namely ALPHAscreen (Perkin Elmer), HitHunter (DiscoveRx), and cGMP HTRF (Cisbio), have become commercially available. All three assays are able to detect femtomolar amounts of cGMP in cell lysates with high specificity rendering sample purification unnecessary. In addition, washing and precipitation steps are omitted resulting in easier handling and increased sample throughput. ALPHAscreen, HitHunter, and HTRF can be used in 96, 384, and 1,536 well plates and should meet most demands with respect to sensitivity and sample throughput. The question which of these assays is appropriate for the respective application is not easy to answer and depends presumably on the existing data acquisition hardware. Although available at a reasonable price and kit size, HTRF requires more sophisticated plate readers making this assay unfeasible for laboratories, not equipped with the appropriate hardware. The requirements for ALPHAscreen with respect to plate readers are lower. However, the available kit size of at least 10,000 test points (and the corresponding price, especially in combination with the necessary Protein A kit) might be oversized for many applications. In contrast, HitHunter is available at a smaller kit size (see Table 1) and generates a chemiluminescence signal that should be detectable with standard luminescence counters. Furthermore, it might be possible to adapt the assay to standard filter-based plate readers by using commercially available chromogenic β-galactosidase substrates, albeit the sensitivity of the assay could be reduced. The fact that HitHunter is the only competition-based immunoassay that offers a gain-of-signal read-out is an additional advantage making this assay a good choice for processing large numbers of samples. Nevertheless it has to be taken into account that although these novel assay formats represent a promising alternative to established methods, there are currently no independent publications available to support the potential feasibility of these assays for the biochemical detection of cGMP aside high throughput screening applications.

4 Concluding Remarks

During the last four decades, a broad range of assays have been conceived and established pushing the boundaries of cGMP detection with respect to sensitivity and sample throughput to the technical limit of the respective time. Some of the described approaches for the biochemical detection of cGMP are no longer used as they offer only limited sensitivity, are laborious, and allowed only the processing of a small number of samples. However, to the respective time, these assays represented state-of-the art approaches and are worth to be mentioned to describe the step-wise evolution of the biochemical cGMP detection systems from the first thin layer chromatography to actual high throughput approaches.

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Novel Techniques for Real-Time Monitoring of cGMP in Living Cells

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Abstract Recent developments of biophysical and electrophysiological techniques have enabled researchers to monitor levels of free intracellular cGMP in real-time and in intact living cells. These techniques are based on the use of cGMP sensors, which respond to cGMP with changes in transmembrane ion current or changes in fluorescence. Here, we describe the principles of these techniques, compare them in terms of sensitivity and discuss possible application for current cell biology and physiology.

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Abbreviations

CFP, enhanced cyan fluorescent protein; cGK, cGMP-dependent protein kinase; CNBD, cyclic nucleotide-binding domain; CNGC, cyclic nucleotide-gated channel; cpGFP, circularly permuted enhanced green fluorescent protein; FRET, fluorescence resonance energy transfer; GC, guanylyl cyclase; PDE, phosphodiesterase; RIA, radioimmuno assay; YFP, enhanced yellow fluorescent protein

1 Introduction

cGMP is a second messenger that regulates numerous cellular events and complex biological processes, including blood vessel tone (Hofmann et al. 2006; Juilfs et al. 1999; Rybalkin et al. 2003), neuronal excitability (Feil et al. 2005; Pfeifer et al. 1999), epithelial electrolyte transport (Hofmann et al. 2006; Jain et al. 1998; Lincoln et al. 1995), phototransduction in the retina (Arshavsky et al. 2002; Leskov et al. 2000; Pugh and Lamb 1993), cell adhesion (Hoffmeister et al. 2008; Massberg et al. 2004) and many others.

cGMP is produced in cells by two types of guanylyl cyclases (GC). Some natriuretic peptide receptors serve as membrane-bound or particulate GCs (Kuhn 2003; Potter and Hunter 2001). These receptors consist of three distinct domains: extracellular domain, transmembrane region and intracellular domain. The extracellular domain binds peptide ligands, members of the natriuretic peptide family (ANP, BNP and CNP), which induces a conformational change activating through the transmembrane helix, the intracellular domain of the receptor containing GC, which starts to synthesize cGMP (He et al. 2001). The second type of GCs is the soluble GC (Friebe et al. 2007; Pyriochou and Papapetropoulos 2005), which is located in the cytosol of many mammalian cells. This enzyme is a heterodimeric protein consisting of α- and β-subunits, and expression of both subunits is required for catalytic activity (Buechler et al. 1991; Harteneck et al. 1990; Kamisaki et al. 1986; Russwurm and Koesling 2004). Each subunit has an N-terminal regulatory domain and a C-terminal catalytic domain that shares sequence homology with the corresponding domains in membrane-bound GC and adenylyl cyclases (Chinkers et al. 1989; Krupinski et al. 1989). Soluble GC is activated by nitric oxide (NO), a paracrine vasorelaxant initially termed EDRF, for endothelium derived relaxing factor (Arnold et al. 1977a, b; Furchgott and Zawadzki 1980; Ignarro et al. 1987).

The effects of cGMP in cells are exerted by activation of three different types of effector molecules: cGMP-dependent protein kinase (cGK) (Hofmann et al. 2006; Schlossmann and Hofmann 2005), cGMP-regulated isoforms of phosphodiesterase (PDE) (Bender and Beavo 2006; Conti and Beavo 2007; Kass et al. 2007; Omori and Kotera 2007) and cyclic-nucleotide-gated ion channels (CNGC) (Craven and Zagotta 2006; Kaupp and Seifert 2002). Activation of all these proteins can be used to monitor changes in intracellular cGMP levels by real-time methods.

2 Classical Methods to Measure Intracellular cGMP

The classical biochemical approach to measure cGMP is based on radioimmuno assay (RIA). This is a destructive method, which requires a lysed batch of cells or tissue where GMP-concentrations can be determined using cGMP antibodies and radioactive cGMP. In addition to RIA, an ELISA method to measure cGMP has been introduced, which is based on the competition between cGMP and a cGMP tracer (e.g. cGMP linked to an acetylcholinesterase molecule). Although RIA and ELISA are sensitive methods, which allow to detect cGMP averaged over a batch of cells, these techniques require the destruction of the sample, they do not allow to monitor the physiologically relevant *free* cGMP levels, and they have very low temporal and no spatial resolution. The need for real-time cGMP measurements with high spatial resolution at the subcellular level is particularly dictated by the concept of cGMP compartmentation, which assumes different cGMP concentrations in various cellular compartments that are ultimately linked to the physiological functions (for review see (Fischmeister et al. 2006)).

3 Cyclic Nucleotide Gated Channels as the Sensors for cGMP

One type of intracellular effector proteins for cGMP is CNGCs, which are present in the retina and olfactory epithelium. These non-selective cation channels are comprised of four subunits, each with one intracellular cyclic nucleotide binding domain (CNBD) for cAMP and cGMP (Biel et al. 2002; Craven and Zagotta 2006; Kaupp and Seifert 2002). In the retina, CNG3 channels are kept open by high cGMP levels in the dark. The PDE6-mediated decrease of cGMP upon rhodopsin activation by light leads to a rapid closure of the channels and thus to hyperpolarization of photoreceptor cells (Arshavsky et al. 2002; Leskov et al. 2000; Pugh and Lamb 1993).

A real-time technique for cyclic nucleotide measurements in living cells has been developed based on olfactory CNG2 channels, which bind both cGMP and cAMP, with a slight selectivity for cGMP (Rich et al. 2000, 2001) (see Table 1). α-Subunits of these channels can be transfected into cells where they form functional homomultimeric complexes integrated into the plasma membrane. Increases in submembrane levels of cGMP open the channels, and this can be monitored by electrophysiological recording of the cation current or by calcium imaging (Fig. 1A).

3.1 Electrophysiological Recordings

CNGCs as membrane-localized sensors can effectively measure cGMP-concentrations at the plasma membrane. These sensors do not undergo desensitization, are not voltage-sensitive and respond rapidly to cGMP. However, cGMP-mediated activation of the channels leads to calcium influx, which might trigger some intracellular

Table 1 Sensitivity of different real-time sensors for cGMP. Reported EC_{50}-values for cAMP and cGMP and maximally measured changes in FRET are presented

Sensor	EC_{50} cGMP	EC_{50} cAMP	Selectivity cGMP/cAMP	ΔFRET max, %	References
CNGCs					
Wildtype CNG A_2	1.6 μM	36 μM	22.5	–	(Rich et al. 2000; 2001)
cGKIα-based FRET sensors					
CGY-Del1	0.02 μM	0.15 μM	7.5	24	(Nikolaev et al. 2006b; Sato et al. 2000)
Cygnet2.1	1.7 μM	>1,000 μM	>600	40	(Honda et al. 2001)
cGi-500	0.47 μM	>1,000 μM	>2,000	77[a]	(Russwurm et al. 2007)
cGi-3000	3.06 μM	>1,000 μM	>2,000	72[a]	
cGi-6000	5.64 μM	>1,000 μM	>2,000	57[a]	
Single-domain FRET sensors					
cGES-DE2	0.9 μM	115 μM	128	40	(Nikolaev et al. 2006b)
cGES-DE5	1.5 μM	630 μM	420	40	
Non-FRET FlincG sensors					
αFlincG	0.035 μM	40 μM	1,140	–	(Nausch et al. 2008)
βFlincG	1.1 μM	48 μM	30		
δFlinc	0.170 μM	48 μM	280		

[a] ΔFRET max measured for the cGi-500 sensor in living cells was ~40%.

events. To overcome possible feedback effects caused by calcium, it is possible to measure the monovalent cation current (Rich and Karpen 2002; Rich et al. 2001).

CNGCs and their mutants with an increased sensitivity for cAMP were initially used to measure cAMP, and these studies supported the concept of compartmentation. In rat cardiac myocytes, β-adrenergic stimulation caused a compartmentalized cAMP signal distributed inside distinct subsarcolemmal domains (Rochais et al. 2004). Such membrane-confined cAMP signaling has been more recently directly visualized using FRET-sensors (Nikolaev et al. 2006a). Later, the same group went on to analyze cGMP dynamics in cardiac cells using the wild type CNG2 channels as sensors for cGMP. The authors tested whether the well known differences between the action of NO-donors and natriuretic peptides in the heart might be due to differential compartmentation of cGMP produced by the two types of GC. Indeed, the particulate pool of cGMP produced after natriuretic peptide stimulation was readily accessible at the plasma membrane and controlled exclusively by PDE2 activity, whereas cGMP produced by the soluble GC had no access to the membrane unless PDE5 was inhibited (Castro et al. 2006). Differential patterning of cGMP responses to NO-donors and natriuretic peptide was also a topic of another study (Piggott et al. 2006), which used CNGCs and measured intracellular calcium.

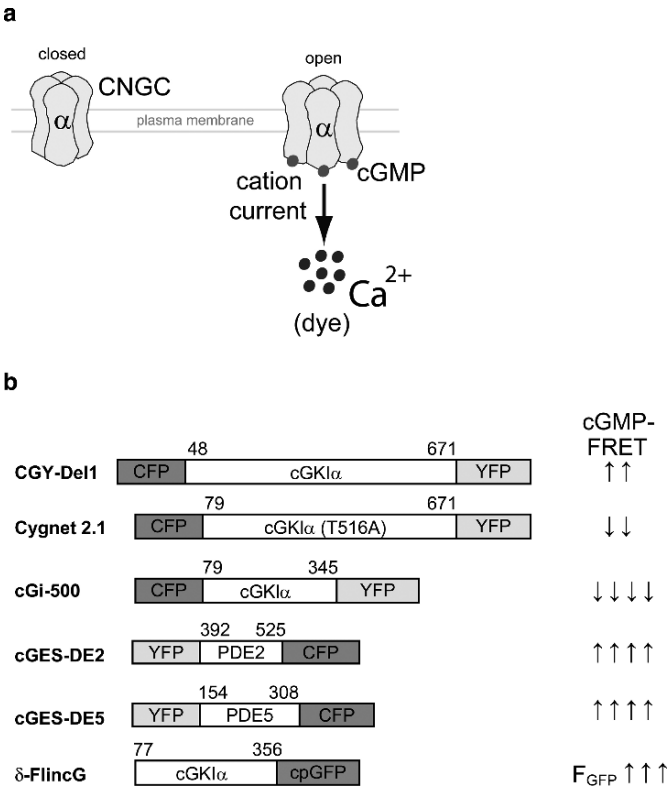

Fig. 1 Schematic structure and working principles of cGMP sensors based on CNGCs, FRET and cpGFP. **a**. Olfactory CNGCs are gated by subsarcolemmal pools of cGMP. Increases in cGMP result in a cation current, which can be measured electrophysiologically or by imaging intracellular calcium, e.g. using cell-permeable dyes. **b**. FRET-based cGMP sensors are constructed using parts of cGKIα or single GAF-domains of PDE2 and PDE5 fused between CFP and YFP. In these chimeric proteins, cGMP induces a conformational change which leads to an increase or a decrease in distance between the CFP and YFP monitored as a change in FRET. In case of FlincGs, the conformational change in the cGKI is transduced to the cpGFP leading to a change of its fluorescence intensity

3.2 Measurements Using Calcium-Sensitive Dyes

Another possibility to measure intracellular cGMP is to use a calcium-sensitive fluorescent dye, e.g. fura-2. A study by Rich and colleagues used HEK293 and vascular smooth muscle cells heterologously expressing CNG2 subunits and labelled them with fura-2 to measure cGMP-dependent calcium increases (Piggott et al. 2006). In these cells, ANP and NO-donors again showed different effects on intracellular cGMP. Particulate GC activated the second messenger production more readily than soluble GC, whose signal was highly confined to the cytosol even in the presence of the non-selective PDE inhibitor IBMX. In contrast, under PDE inhibition total

cGMP levels in both compartments were equally high, and so the authors concluded that natriuretic peptides and NO-donors stimulate cGMP synthesis in different compartments of the cell (Piggott et al. 2006).

Another calcium sensor used to image cGMP-dependent CNGC activity is the photoprotein aequorin (Wunder et al. 2005a, b, 2007). Calcium influx through cGMP-gated CNGCs leads to an increase in aequorin luminescence in the presense of appropriate co-factors (such as coelenterazine), which can be measured immediately after addition of compounds increasing cGMP levels. In this case, the reporter cell line is generated, which stably expresses the soluble GC, CNGCs and aequorin. The advantage of this system is a high signal-to-noise ratio and the possibility to use it in the pharmaceutical industry for high-throughput screening of such compounds as PDE inhibitors. In this case, the reporter cell line must be transfected with the PDE of interest (Wunder et al. 2005a, b). This method has been also used an as indirect assay to measure NO-production (Wunder et al. 2007).

4 FRET-Based Techniques

During the last decades, microscopic techniques have been developed for the real-time monitoring of biochemical events in living cells including the dynamics of the second messengers' calcium, cAMP and cGMP. Many of these techniques are based on fluorescence resonance energy transfer (FRET). FRET is a quantum mechanical phenomenon that occurs between a fluorescence donor and a fluorescence acceptor that are in molecular proximity of each other, provided that the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. FRET is very sensitive to the distance between the two fluorophores and thus allows the monitoring of changes in their distance that may occur either when they are attached to a pair of interacting proteins (*inter*molecular FRET) or when they are localized in a single protein that changes its conformation (*intra*molecular FRET). Examples of conformationally sensitive intramolecular FRET include sensors for the second messengers calcium and cyclic nucleotides (see below) as well as fluorescently labeled G-protein-coupled receptors that sense agonist activation (Vilardaga et al. 2003; Lohse et al. 2007). Examples of intermolecular FRET include cAMP-sensors based on the dissociation of the subunits of cAMP-dependent protein kinases (Zaccolo 2004; Mongillo et al. 2004) as well as those monitoring the activation state of G-proteins (Bünemann et al. 2003) or the interaction between receptors and G-proteins (Hein et al. 2005).

This methodology has been used to create a vast variety of fluorescent sensors (see the following review articles for details Giepmans et al. 2006; Miyawaki 2003; Lohse et al. 2008; Zaccolo 2004; Zhang et al. 2002). These approaches not only permit a detailed investigation of the kinetics of intracellular signaling, but they also permit the imaging of local changes in second messengers and, thus, the study of signaling compartments in intact cells (Fischmeister et al. 2006; Nikolaev et al. 2006a; Rich and Karpen 2002). Using appropriate sensors it is even possible to

image two second messengers simultaneously, such as calcium and cAMP (Landa et al. 2005). FRET has been applied in various approaches for cGMP measurements by developing and using FRET-based sensors, which report the activation of cGKs or cGMP-regulated PDEs.

4.1 CGY-Sensors Based on the Full-Length cGKIα

The first approach to measure real-time changes in intracellular cGMP has been developed using cGKIα as a backbone for a FRET monomolecular sensor (Sato et al. 2000). This sensor named CGY-Del1 is based on the well-established fact that the cGKI protein changes its overall conformation upon cGMP binding to the two sites in the regulatory domain, which results in an increase in FRET between CFP and YFP (Fig. 1B). In the CGY-Del1 sensor only 47 amino acids on the extreme N-terminus are truncated to prevent the dimerization of the cGKIα. However, the sensor includes the autoinhibitory region of the cGKIα present on the N-terminus, which might account for a very high sensitivity to cGMP of about 20 nM (see Table 1). Unexpectedly, such N-terminal deletion resulted in an almost complete loss of cGKIα selectivity towards cGMP, and cAMP activates this sensor half-maximally at 200 nM, making selective cGMP measurements difficult, because intracellular cAMP concentrations normally far exceed those of cGMP.

Recently, the same group has developed an approach for real-time NO-measurements using CGY-Del1 genetically coupled to α and β subunits of the soluble GC (Sato et al. 2005). This construct serves as an amplifier-coupled fluorescent indicator for NO and is capable of visualizing physiological nanomolar dynamics of NO in living cells with a detection limit of 0.1 nM. This approach demonstrated that 1 nM of NO, which is enough to relax blood vessels, is generated in vascular endothelial cells even in the absence of shear stress (Sato et al. 2005). More recently, the CGY-Del1 sensor has been used to create a reporter cell line, which can be used to monitor NO levels in the co-culture with the cells of interest. For this purpose, cells from a pig kidney-derived cell line PK15, which endogenously expressed soluble GC, are transfected with the CGY-Del1 sensor. The resultant reporter cell line called Piccell showed an outstanding sensitivity to NO and uncovered an oscillatory release of picomolar concentrations of NO from hippocampal neurons. Furthermore, the authors demonstrated that calcium oscillations in hippocampal neurons underlie the oscillatory NO release from the neurons during neurotransmission (Sato et al. 2006).

4.2 Cygnets Based on cGKIα

In parallel to the development of the CGY-Del1 sensor, another group headed by Wolfgang Dostmann generated cGKIα-based sensors for cGMP, which were slightly shorter on their N-terminus and did not include the autoinhibitory region

(Fig. 1B) (Honda et al. 2001). This might be the reason for the fact that these sensors called *cygnets* retained the exceptional selectivity of cGKIα for cGMP (Table 1). Cygnets reacted to natriuretic peptides and NO-donors in cell lines and Purkinje cells with cGMP-concentration-dependent decreases in FRET, allowing for real-time spatial and temporal analysis of this second messenger. In addition, the authors introduced a T516A point mutation to inactivate the catalytic domain of the kinase to prevent the down-stream intracellular events (Honda et al. 2001).

Cygnet 2.1 has been used in several studies to analyze real-time dynamics of cGMP in cultured vascular smooth muscle cells (Cawley et al. 2007; Honda et al. 2005a, b; Sawyer et al. 2003), rat neonatal cardiomyocytes (Mongillo et al. 2006; Takimoto et al. 2005), mouse teratocarcinoma cells (Ma and Wang 2007) and in thalamic neurons (Hepp et al. 2007).

In aortic smooth muscle cells transfected with cygnet 2.1, different NO-donors induced concentration dependent, transient cGMP responses irrespective of their rates of NO release. The kinetics of such cGMP peaks were governed by the concerted action of the soluble GC and PDE5, involving the cGKIα dependent phosphorylation of PDE5. Interestingly, cGMP transients could be elicited repeatedly without apparent desensitization of GC or by suppression of cGMP via long-term PDE5 activity, supporting the phasic nature of smooth muscle physiology (Cawley et al. 2007).

Measurements of cGMP in neonatal cardiac myocytes have further supported the idea of cGMP compartmentation. Inhibition of PDE5 with sildenafil enhanced the cGMP production and this effect was sensitive to the inhibition of NO synthase (NOS3), which might be coupled to the catabolic regulation of cGMP by PDE5 (Takimoto et al. 2005). Another study using neonatal cardiac myocytes and cygnet 2.1 demonstrated that activation of β_3-adrenergic receptor leads to the eNOS-dependent activation of cGMP synthesis which in turns activates PDE2 serving as a sink for cAMP produced after activation of β_1 and β_2 adrenergic receptor subtypes. Such a feedback regulatory loop is supposed to act in spatially defined signalling domains (Mongillo et al. 2006).

Very recent studies in living thalamic neurons used cygnet 2.1 expressed in brain slices by viral gene transfer (Hepp et al. 2007). In these cells, basal cGMP levels could be increased or decreased by NO-donors or PDE- and GC-inhibitors. Interestingly, PDE2 was shown to regulate the basal cGMP levels since an application of a selective PDE2- but not of PDE1- and PDE10-inhibitors increased the basal cGMP. In contrast, another cGMP-specific hydrolyzing enzyme, PDE9, was implicated in a decrease of cGMP levels after the stimulation with NO-donors, suggesting that different PDE isoforms may play distinct roles in the regulation of basal versus stimulated cGMP levels (Hepp et al. 2007).

4.3 cGi-500-Like Sensors Involving Regulatory Regions of cGKIα

To increase the sensitivity of the cygnet sensor for cGMP and to improve its speed and dynamic range, the group of Doris Koesling has introduced a systematic

approach to create FRET-based cGMP sensors based on cGKIα (Russwurm et al. 2007). The authors truncated a cygnet-like sensor C-terminally to obtain more sensitive constructs. Out of 24 cGMP-responsive constructs, three were selected to cover a range of cGMP affinities with an EC_{50} between 500 nM and 6 μM (Table 1). The most sensitive of these indicators called cGi-500 is a much shorter sensor than cygnet 2.1 because of the deleted catalytic domain (Fig. 1B). Although the larger C-terminal parts responsible for the interaction with the cGMP-binding regions and determining the ligand specificity (Landgraf et al. 1986) are deleted, cGi-sensors have been reported to possess excellent specificity for cGMP in vitro, fast binding kinetics and twice the dynamic range of existing cGMP sensors (Russwurm et al. 2007). These properties make the cGi-sensors the most sensitive FRET-based indicators to date. However, experiments in living cells showed a ∼two-fold lower dynamic range of the sensors with maximal amplitudes of only ∼40% which is comparable to that of cygnet 2.1 (Russwurm et al. 2007). Future studies should demonstrate whether these sensors retain their high selectivity for cGMP in vivo and can be used in physiologically relevant cells.

4.4 FRET Sensors Based on Single GAF-Domains from PDE2 and 5

The low selectivity of the CGY-Del1 sensor and the slow responsiveness of cygnet 2.1 in the physiologically relevant range of cyclic nucleotide concentrations have led our group to attempt the generation of sensitive and small FRET-sensors based on a single cGMP binding domain, as it had been achieved before for cAMP sensors (Nikolaev et al. 2004; Nikolaev and Lohse 2006). Such cAMP sensors have also been used to serve as indirect monitors of cGMP-concentrations. For example, in aldosterone producing cells, cAMP levels are subject to degradation by cGMP-activated PDE2 and are, therefore, regulated by natriuretic peptides (Nikolaev et al. 2005). For the development of cGMP-sensors, we focused on the cGMP binding domains of PDE2 and PDE5, which contains two in-tandem homologous sites of about 110 amino acids located at the N terminus and called regulatory GAF-domains (from c**G**MP-regulated phosphodiesterases, bacterial **A**denylyl cyclases, and transcription activator **F**lhA, where such domains were identified). The amino acid sequence of the GAF-domains is different from that of cGKI and CNGCs. The cyclic GMP binds to these allosteric sites and stimulates PDE activity via a conformational change, which is transduced from the GAF domains to the catalytic region (Martinez et al. 2002). Indeed, if a single cGMP binding GAF-domain was fused between CFP and YFP (Fig. 1A), an increase of FRET in such sensors was observed upon the stimulation of cGMP production in cells (Nikolaev et al. 2006b). This observation led to the development of cGES-DE sensors (see Table 1), which reacted rapidly to cGMP, showed good sensitivities (EC_{50} values in the range of 1 μM) and were well selective for cGMP, as demonstrated in living cells. Even in a cell line with low endogenous amounts of soluble GC, cGES-DE sensors showed robust

repetitive increases in FRET, which were followed by PDE5-dependent decreases of cGMP. In primary mesangial smooth muscle cells, such sensors reported the NO-dependent cGMP signals and could be used for real-time cGMP imaging (Nikolaev et al. 2006b). One important advantage of these sensors compared to the cGKI-based probes is their compactness, the presence of only one binding domain and absence of catalytic and other domains, which might be involved in protein–protein interactions. This might facilitate the use of these sensors for the generation of the transgenic in vivo models.

Very recently, another similar sensor based on the GAF-A domain of PDE5 has been developed to monitor cGMP levels based on bioluminescence resonance energy transfer (BRET) (Biswas et al. 2008). This method is similar to FRET with a difference that the energy is transferred from a donor enzyme, e.g. luciferase whose luminescence is monitored, to a fluorescent acceptor, typically a variant of GFP (Pfleger and Eidne 2006).

At present, a wide palette of FRET-based sensors for cGMP is available, which can be applied for real-time monitoring of this second messenger in living cells. These sensors differ substantially in their sensitivity, kinetic properties, signal-to-noise ratio and selectivity (see Table 1). The choice of the sensor depends on the type of application and the need for high sensitivity and cGMP/cAMP selectivity.

5 FlincGs: Novel Non-FRET Fluorescent Sensors

Recent developments of circularly permutated fluorescent proteins such as cpGFP offered a possibility to visualize the conformational changes in protein by just fusing them to one cpGFP molecule. The measurements are based on the monitoring of cpGFP intensity, which increases upon the change of conformation. This strategy was originally used to create fluorescent calcium sensors (Nagai et al. 2001; Nakai et al. 2001). Such calcium sensors proved useful for imaging in living cells and even in beating hearts in vivo (Tallini et al. 2006).

The group of Wolfgang Dostmann has recently created similar sensors for cGMP called FlincGs (fluorescent indicators of cGMP) (Nausch et al. 2008). Such sensors are comprised of the regulatory cGKIα regions similar in length to the cGi-500 sensor, which are N-terminally fused to the cpGFP. To prevent the dimerization of cGKIα, its extreme N-terminal domain was deleted to create the δFlincG sensor (Fig. 1B). This sensor showed a 1.7-fold increase in cpGFP fluorescence upon full stimulation with cGMP, rapid association and dissociation kinetics, high affinity and selectivity (280-fold cGMP/cAMP preference) for cGMP (see Table 1). Unexpectedly, δFlincG showed a second minor excitation peak at 410 nm, which allows ratiometric excitation measurements. This sensor was expressed in vascular smooth muscle cells and showed rapid kinetics of cGMP in response to various NO-donors. Finally, the authors investigated the previously discussed hypothesis that natriuretic peptides and NO donors stimulate cGMP synthesis in distinct subcellular compartments. For this purpose, they used confocal microscopy and aortic smooth muscle cells transfected with δFlincG using adenovirus. Sustained stimulation of soluble

GC with NO-donors induced transient global cGMP elevations with the decay phase dependent on the activity of PDE5. In contrast, stimulation of the particulate GC with natriuretic peptide elicited sustained submembrane elevations of cGMP which were converted to global cGMP elevations by PDE5 inhibition (Nausch et al. 2008). This highly interesting finding is the first direct optical visualization of cGMP produced in different compartments and supports previous observations in cardiac myocytes (Castro et al. 2006) and smooth muscle cells (Piggott et al. 2006) obtained using CNGCs as the sensor for cGMP.

6 Conclusions and Perspectives

The advent of FRET, fluorescent microscopy and electrophysiology has provided researchers with a variety of new tools to directly monitor rapid subcellular dynamics of cGMP with unprecedented spatial and temporal resolution, which are unobtainable by classical cGMP assays.

A large number of cGMP sensors mentioned in this review show different kinetic properties, sensitivity and selectivity for cGMP (Table 1). They are based on different techniques such as electrophysiology (CNGCs), FRET and simple fluorescence (FlincG). The choice of a sensor for each particular application will depend on the expected basal and stimulated cGMP concentrations in cells, cross-talk between cGMP and cAMP signalling pathways and the microscopic or electrophysiological method available for recording the sensor's signal.

Novel optical techniques, which allow direct visualization of cGMP, will ultimately facilitate our understanding of intracellular dynamics of this second messenger in different cell types, as well as in diverse in vivo models. They should give new insights into cGMP compartmentation as a mechanism of fine regulation of cellular functions.

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Part II
Pharmacology of cGMP

NO and sGC-Stimulating NO Donors

Ian L. Megson and Mark R. Miller

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Abstract Our knowledge of nitric oxide (NO) as a crucial endogenous signalling molecule continues to expand. Many, but not all, of the actions of NO are mediated by activation of soluble guanylyl cyclase (sGC) in target tissues. The aim of this chapter is to encapsulate the functions of NO in mammalian biology, tied to

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the chemistry of this unusual signalling entity. The experimental usefulness and therapeutic potential of the most widely utilised NO donor drugs is reviewed, with special consideration given to the importance of choosing the correct NO donor for any given experiment, in vitro, in vivo or in clinical studies.

Keywords: Nitric oxide · Measurement · NO donors · Organic nitrates · NONOates · Nitrosothiols · NO hybrids

1 Introduction

Nitric oxide (NO) and its interaction with soluble guanylyl cyclase (sGC) has turned our understanding of endogenous signalling on its head: no longer is cell signalling the exclusive domain of large organic or peptide molecules that dock with specific receptors to trigger downstream events. For NO is a small, inorganic free radical that has a very short half-life in biological systems and, whilst sGC is generally regarded to be its primary target, its reactive nature facilitates a wide variety of chemical interactions, accounting for a range of cGMP-independent effects, particularly when generated in large amounts.

NO is now recognised to have crucial roles in the cardiovascular, nervous and immune systems and it is unsurprising that, since its identification as a key mediator in the 1980s, the interest in NO and its biological functions has mushroomed: each year since 1995, there have been >2000 papers that include "nitric oxide" in the title and many more that deal with some aspect of NO. In this chapter, we have concentrated on providing an overview of the different roles of NO as a precursor to discussing the use of NO donor drugs as sGC stimulators in pre-clinical and clinical studies, both as tools for investigating the role of NO and as potential therapeutics. There are now a wide range of NO donors available, each with its own specific properties: selection of the best-suited agent for the purposes of a specific experiment is a critical consideration that is easily overlooked and can have a significant bearing on the results that are obtained and their interpretation.

1.1 Functions of NO

NO is a powerful vasodilator but its actions, both within the cardiovascular system and elsewhere, range well beyond its ability to relax vascular smooth muscle (see Moncada et al. 1991 for comprehensive review). Remarkably, NO is now recognised to behave as a neurotransmitter, neuromodulator, anti-thrombotic agent, pro- or anti-inflammatory agent, modulator of cellular respiration and cytostatic and cytoxic agent, depending on the concentration of NO in the locality, together with its source (Fig. 1).

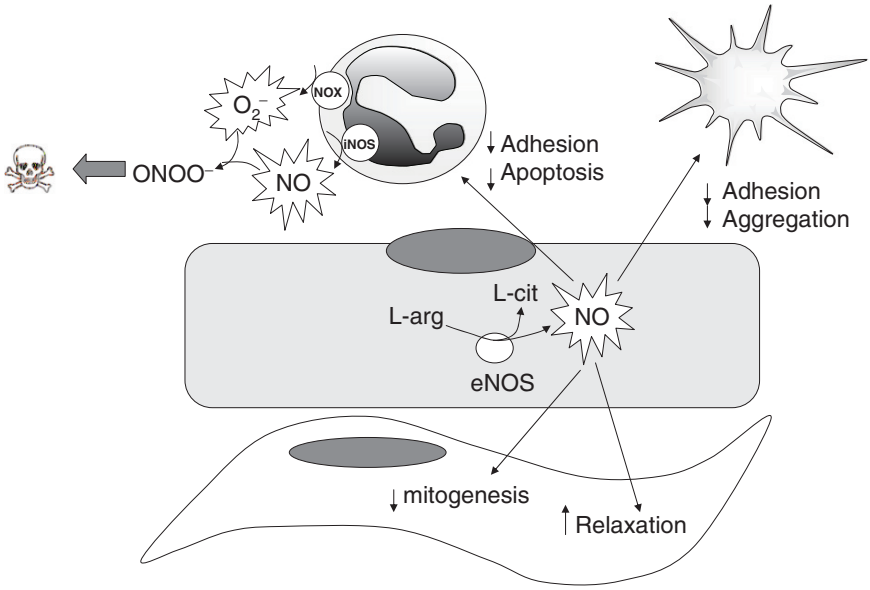


Fig. 1 Sources and biological impact of NO in the cardiovascular system

1.1.1 In the Cardiovascular System

NO is synthesised from L-arginine, primarily through the actions of the endothelial isoform of the enzyme NO synthase (eNOS or NOSIII; Fig. 1). NO synthesised in the endothelium diffuses in three dimensions away from the source and easily crosses plasma membranes on account of its solubility in both lipid and aqueous phases. As it diffuses, so the concentration falls as a result of both the increasing volume of dissolution with distance and through interaction with reactants. Thus, endothelium-derived NO causes vascular relaxation through interaction with sGC in the blood vessel wall, but also diffuses into the lumen of the vessel where it interacts with a number of other cell types (Fig. 1). There is a fundamental barrier to the effectiveness of luminal NO in reaching target cells on account of the very high concentration of hemoglobin, which would be anticipated to rapidly scavenge it all and prevent physiological effects (Lancaster 1996; Butler et al. 1998). However, given that endothelium-derived NO apparently can influence the function of blood-borne cells in vivo, it has been postulated that encapsulation of hemoglobin in red blood cells, coupled with their separation from the endothelium by so-called plasma-streaming in conduit arteries, is sufficient to facilitate at least some NO reaching target cells (Lancaster 1996; Butler et al. 1998). As with smooth muscle, the evidence suggests that sGC is the primary target for NO in both platelets (which also produce their own NO upon activation) and inflammatory cells (some of which also generate high levels of NO in response to inflammatory stimuli – see below), but there is increasing evidence to suggest that sGC-independent effects play a part in

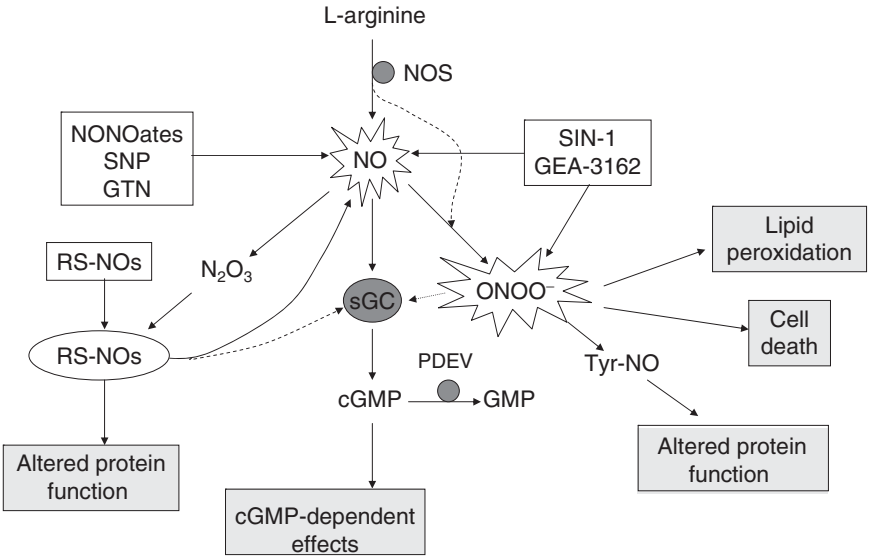


Fig. 2 sGC-dependent and –independent functions of NO: sites of activity for NO donor drugs

the inhibition of platelet function at least (Homer and Wanstall 2003; Crane et al. 2005). The potential effects and fates of NO are shown in Fig. 2.

1.1.2 In the Nervous System

The neuronal isoform of NO synthase (nNOS, bNOS, NOS I) is found in both the central and peripheral nervous systems. In the central nervous system, nNOS is primarily found post-synaptically, where it appears to have a neuromodulatory function that is associated with long-term potentiation and memory. In the peripheral nervous system, nNOS is primarily found in pre-synaptic nerve terminals in the enteric nervous system that largely supplies the abdominal organs. Although implicated in functions such as gastric emptying, NO is most famously recognised in this setting for its ability to relax corpus carvernosal tissue and mediate penile erection. In both settings, the implication is that sGC is the primary target, but there is the likelihood that sGC-independent effects are also important.

1.1.3 In Inflammation and the Immune System – sGC-Independent Effects

Induction of the inducible isoform of NO synthase (iNOS; NOSII) and the subsequent generation of relatively high (μM) local concentrations of NO, in conjunction with other noxious agents, superoxide from NAD(P)H oxidases (forming peroxynitrite, $ONOO^-$) and hyperchlorous acid from myeloperoxidase in some leukocytes

(e.g. neutrophils), is central to the first-line defence against microbial invasion. This isoform is not regulated by calcium and therefore has a high level of activity. The role of NO and NO-related agents in this arena is to kill invading pathogens through poisoning of the respiratory chain and DNA damage (Fig. 1).

Any cell has the potential to express iNOS and very severe infections (e.g. toxic shock syndrome) can result in systemic iNOS expression, generating large quantities of NO that, together with cytokines, induce irreversible vasodilatation and potentially fatal hypotension. Chronic iNOS induction is associated with many conditions, including arthritis, atherosclerosis, diabetes and bronchial diseases. In this setting, iNOS is generally regarded to be harmful and to contribute to disease progression: a failed counter-regulatory system?

2 Chemistry of NO

Although NO is a free radical, it is a relatively unreactive one. This is an important consideration because, were it more reactive, its activity would be totally indiscriminate and it would be a highly cytotoxic agent with properties similar to that of hydroxyl anion ($^{\cdot}OH$). Nevertheless, the biological half-life of NO is usually quoted as 1–3 s (Palmer et al. 1996), a pre-determining factor in the need for *de novo* synthesis of NO on demand, as opposed to its deployment in storage vesicles that are released upon stimulation. The lack of toxicity of NO under physiological conditions is primarily governed by the extremely low levels of release on account of highly regulated enzymes responsible for its synthesis (eNOS, nNOS). A captivating aspect of NO is the switch from a constitutive, highly regulated, protective agent and neurotransmitter to a highly cytotoxic agent (from iNOS) that is used by inflammatory cells as part of their armoury against pathogenic invasion.

2.1 Interactions of NO with sGC

The primary target for NO when acting as a signalling molecule is recognised to be sGC, wherby binding of NO to the heme iron results in activation of the enzyme and generation of cGMP from GTP (see Waldman and Murad 1988 for comprehensive review). Details surrounding the NO-sGC interaction and activation processes within the enzyme are covered elsewhere in this book.

2.2 Interaction with Other Heme Groups

The high affinity of heme iron (in oxidation state II) for NO ensures that hemoglobin is a substantial sink for NO, particularly when NO is released abluminally.

The precise reaction that takes place is dependent on both the oxidation state and the oxygenation state of the iron. Reduced, deoxygenated heme iron has a high affinity for NO and reacts to form a relatively stable iron-nitrosyl, which is detectable by electron paramagnetic resonance (EPR), although the chemistry underlying formation of this species has been shown to be considerably more complex than a straight nitrosylation reaction (Han et al. 2002). In the presence of oxygen, the consensus is that NO is more likely to react with O_2 at the heme centre to generate nitrate and met-Hb (iron III; Fig. 3). The latter will still react with NO, but has a much lower affinity than Fe(II) heme. Whilst hemoglobin represents the most abundant heme-containing protein, there are many other examples that represent legitimate targets for NO interaction, including NO synthases (Abu-Soud et al. 2000) and cyclo-oxygenases (Mollace et al. 2005). Furthermore, respiratory enzymes contain iron-sulfur centres that are also targeted by NO and $ONOO^-$, leading to inhibition of respiration. The interaction of NO with heme proteins and other metalloproteins is reviewed by Thomas et al. 2003.

2.3 Reaction with Oxygen

The reaction of NO with oxygen in solution (Fig. 3) provides a good example of the impact of NO concentration on reaction rate. The kinetics of the reaction of NO with O_2 are second order with respect to NO and pseudo-second order overall (Lewis and

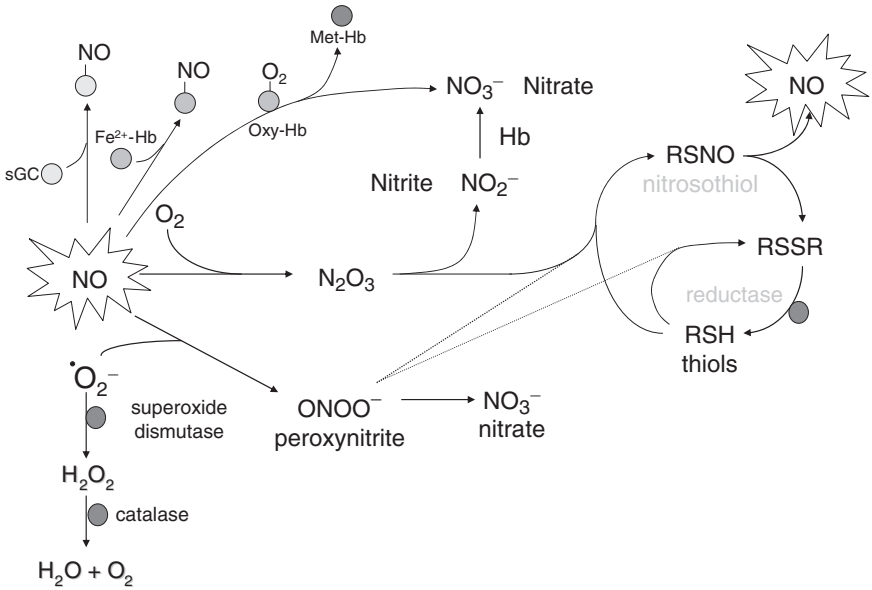


Fig. 3 Interaction of NO with biologically important entities

Deen 1994). Although the rate constant for the reaction is high $(6 \times 10^6\,\mathrm{M}^{-2}\,\mathrm{s}^{-1})$, the actual half-life of NO in an oxygen saturated solution containing nothing else is highly dependent on the NO concentration. Thus, the half-life for NO under these conditions ranges from milliseconds in the mM range to minutes in the micromolar range (Hakim et al. 1996) and hours in the nanomolar (physiological) range. The lack of correspondence between these figures and the estimated half-life of NO in the biological setting (seconds), is indicative of the relative importance of other reactions in tissue.

2.4 Reaction with Reactive Oxygen Species

Not surprisingly, the reaction of NO with other radicals has the potential to be faster than that with oxygen. The reaction of NO with superoxide anion $(^{\bullet}O_2^{-})$ is the best-studied (Fig. 3; comprehensively reviewed in Crow and Beckman 1995); $^{\bullet}O_2^{-}$ is generated from the respiratory chain, many enzyme processes and in activated inflammatory cells, so it holds considerable importance in biology. The rate constant for this reaction is so high $(6 \times 10^9\,\mathrm{M}^{-2}\ \mathrm{s}^{-1})$ that the rate of diffusion is more limiting than the reaction itself (i.e. it is diffusion-limited). Were $^{\bullet}O_2^{-}$ the only reactant for NO, the half-life of 1 nM NO in 10 nM $^{\bullet}O_2^{-}$ would be in the order of 10 ms, despite the low concentration of reactants present (and ignoring the impact of diffusion on the reaction).

2.5 Interaction with Thiols

NO itself is a poor nitrosating agent for thiols, but many of the products of oxidation of NO (e.g. N_2O_3, N_2O_4 and $ONOO^-$) react with thiols in amino acids, peptides and proteins in vivo to generates S-nitrosothiols (Fig. 3). Nitrosation of thiols is now recognised not only to generate potential NO donating species, but also to alter protein and enzyme structure and function; it has long been suggested that nitrosation of critical residues in sGC itself alters the sensitivity of the enzyme to NO binding at the heme site.

3 Measurement of NO

NO is notoriously difficult to measure on account of the many reactions that it might undergo in a given medium. Furthermore, direct measurement is all but impossible on the basis that NO is not only short-lived, but is usually generated in very small amounts and has no distinctive characteristics to facilitate easy measurement (e.g. a unique ultraviolet or visible spectrum). As a result, we are almost always restricted

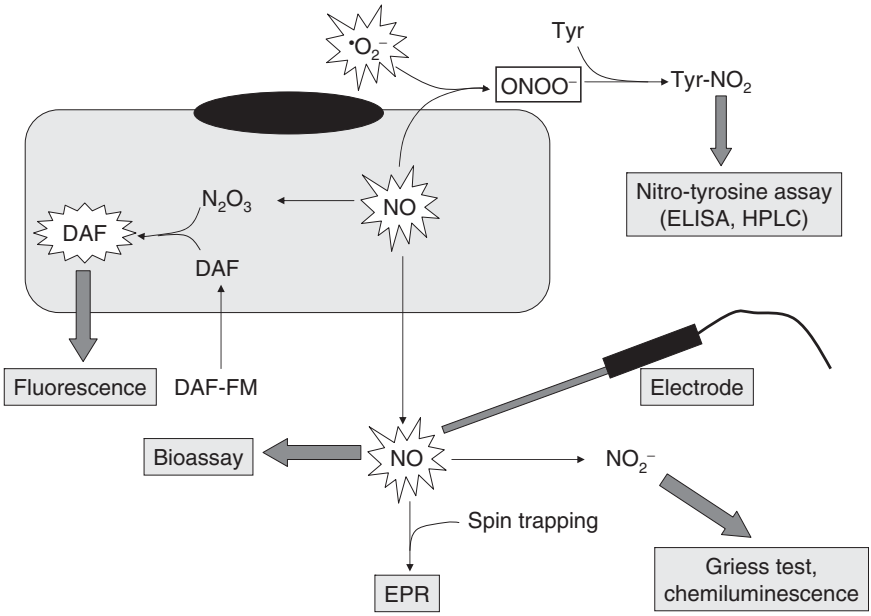


Fig. 4 Techniques available for measurement of NO

to measuring a surrogate marker of NO, be it the oxidation products (e.g. nitrite, nitrate) or the measurable product of the reaction of NO with another agent (e.g. a spin trap for electron paramagnetic resonance, ozone for chemiluminescence, a fluorescent dye or at an electrode surface to generate an electrical signal). Which one of these methods one chooses is determined by the specific experiment undertaken, but none can be regarded as a perfect solution to the issue. Some potential means of measuring NO and related species are shown in Fig. 4.

3.1 NO Electrodes

Measuring NO via an electrode is as close as one can get to direct measurement. A number of different electrodes are commercially available, but they all work on the principle of NO reacting at an electrode to release electrons to the circuit, which can subsequently be measured as current. The primary advantages over almost all other methods of NO detection are that NO consumption is minimal and responses are real-time.

The keys to the success of electrodes are (a) their specificity for NO and hence avoidance of non-specific signals and (b) the loss of NO after reacting at the electrode surface. Different strategies are used to achieve these ends: with the Clark-type electrode, a gas-permeable membrane goes some way to selecting NO for reaction

at the electrode surface and the voltage bias at the reference electrode (+860 mV) is specific for NO (a different bias could be used to select for O_2, for example). The NO ultimately undergoes an irreversible reaction mediated by OH^- in the electrolyte to generate $NO_2{}^-$, which does not generate a signal. Porphyrinic sensors, on the other hand, make use of the specific reaction of NO with metals (Fe or Ni in particular) to generate the current at the electrode surface (Malinsky and Taha 1992). The sensitivity of electrodes has improved markedly and a number of suppliers now claim sensitivity down to low nM concentrations (Zhang 2004). Indeed, experiments utilising a Clark-type electrode in tandem with conventional myography (Miller et al. 2008) suggest that electrodes are approaching the exquisite sensitivity of tissue.

Electrodes have been used successfully and extensively in measuring NO generation from NO donor drugs, where conditions can be highly controlled; their use in tissue culture and *in vivo*, though possible, is fraught with practical difficulties.

3.2 Nitrite and Nitrate Measures

The Griess test has long been the mainstay for measuring nitrite. The test relies on the conversion of colourless sulphanilamide and N-(1-naphlyl)ethylendiamine to a purple azo dye (absorption peak 450 nm). It can be used to measure nitrate + nitrite (often described as NO_X) by pre-reduction of nitrate, either by nitrate reductase or through chemical reduction. Although such measures have been successfully used to show the accumulation of NO-derived oxidation products under experimental conditions, until recently, the lack of sensitivity of the assay has been a serious drawback – for example, it is not sufficiently sensitive to measure plasma nitrite (100–500 nM) as a measure of endothelial function *in vivo*. A recent advance has greatly enhanced the sensitivity, however, whereby HPLC is used in Griess analysis (Tsikas 2007). It will be interesting to see how this development impacts on the utilisation of this simple assay in the future.

3.3 Chemiluminescence

One of the most enduring techniques for measuring NO is through its reaction with ozone to produce a chemiluminescent product (Palmer et al. 1987). The assay involves blowing NO generated in solution into the headspace and on into the chemiluminescent analyser in a stream of N_2 gas. The NO in this setting can be generated from NO donor drugs in physiological media or from nitrite in biological fluids as a surrogate for *in vivo*, cell or tissue NO generation. Chemiluminscence has also gained recognition for measuring S-nitrosothiols through metal ion-mediated release of NO and it is easily adapted to measure nitrite as a surrogate for NO generation *in vivo* and in tissue culture; in this situation, the nitrite-containing sample is

injected into an acid-containing mixture in the reaction vessel in order to generate NO for measurement (Pelletier et al. 2006).

3.4 Fluorescence

A number of fluorescent markers for NO have been generated (e.g. 4,5 diamino fluoroscein, DAN; Suzuki et al. 2002). Such markers have great potential in the NO field, particularly as they promise both localisation (using diacetate forms) and quantification, but current proponents are unfortunately limited by their requirement for higher oxides of nitrogen for activiation. Thus, these agents are really markers for nitrosating agents (perhaps useful in its own right) rather than NO *per se*. Care must therefore be exercised in interpreting results using these agents.

3.5 Electron Paramagnetic Resonance (EPR)

EPR is a means of detecting paramagnetic species, i.e. those with unpaired electrons. However, NO is too short-lived and has too broad a spectrum to be detected satisfactorily by EPR. Instead, NO can be trapped by so-called spin-traps, generating stable paramagnetic species that can be detected by EPR. The limitation of EPR surrounds the specificity of the spin-traps so far available: perhaps the most suitable for NO research are heme-containing compounds (including hemoglobin) and iron complexes of dithiocarbamates, which have been used with some success in this arena (Kleschyov et al. 2007).

4 NO in Health and Disease

NO was originally identified as an endothelium-derived relaxing factor (EDRF; Furchgott and Zawadzki 1980) and its action as a powerful vasodilator is very well-documented. However, it transpired that NO has a wide range of activities other than vasodilatation, both within the context of the cardiovascular system and elsewhere.

4.1 Cardiovascular Impact – an Anti-Atherogenic Agent

Historically, NO was identified as a very powerful endogenous vasodilator (hence "relaxing" factor, EDRF). However, it emerged, that, whilst inhibition of endogenous NO succeeded in increasing blood pressure, the effect was perhaps smaller than had been anticipated. It has since emerged that the influence of NO as an EDRF

is not equivalent in all blood vessels – in general its impact is greater in large vessels, dilatation of which only has a modest impact on systolic blood pressure, largely through changes in compliance. Why then, is the primary function of NO focused on large conduits that have little impact on blood pressure? The answer might lie in the fact that large conduit arteries are prone to atherosclerosis, the disease process that underpins plaque development and luminal occlusion that can result in heart attacks and strokes. It transpires that NO is central to a number of other functions that help to inhibit the atherogenic process and that endothelial dysfunction and the consequent loss of NO is a key trigger for atherogenesis.

There is an enormous literature on the vasodilator, anti-platelet and anti- (and pro-) inflammatory effects of NO, in a wide range of studies from cell culture through to human clinical studies. Many have investigated the role of NO through inhibiting NO synthase(s) with pharmacological tools (N^G-monomethyl-L-arginine – L-NMMA; N^{ω}-nitro-L-arginine methyl ester – L-NAME) and in specific knockout mice (e.g. iNOS, eNOS). However, the alternative approach is to supplement endogenous NO with donor moieties that provide exogenous NO (so-called NO donor drugs).

5 NO Donors – Which One Should I Use?

Given the importance of NO in such a wide range of health issues, there has been an enormous growth in the use of exogenous NO to examine its effects in cells, tissues, whole animals and humans. The growth in experimental research in this field has been mirrored by the proliferation of agents that can deliver NO, each with their own very specific features. In designing an experiment that is to involve NO delivery, one is faced by a critical decision that is not trivial and is fundamental to the interpretation of the results: "which NO donor?" The answer that you arrive at should be dictated by the responses to a number of considerations relating to the specific experiment that is to be undertaken. In selecting an NO donor for a specific experiment, it is important to recognise that the compound that you choose is unlikely to be perfect for the application, nor can the results be regarded as "typical" for NO donors in general: each has a unique profile with respect to NO species generated, tissue penetration, and site and mechanism of NO release.

5.1 NO Gas

In the years that followed the discovery of EDRF, there ensued a race to be the first to formally identify it. NO emerged as an unlikely early front-runner, as confirmed by experiments that compared the characteristics of "authentic" NO (gas in solution) with EDRF. Indeed, the studies that finally confirmed the identity of EDRF as NO used a chemiluminescent analyser to detect EDRF against an NO standard.

Since this early application for solutions of NO in the identification of EDRF, its use in pre-clinical studies has all but disappeared, mainly on account of the difficulty in handling NO gas and, most notably, preventing its reaction with oxygen to form higher oxides of nitrogen. Even with the most careful of handling and the best efforts to remove oxygen and contaminating oxides of nitrogen, it is likely that NO delivered in this manner is really a mixture of NO, NO_2, N_2O_3 and nitrite, which could have an important impact on the interpretation of results obtained. In the clinical setting, however, inhaled NO has evolved as one of the few real therapeutic advances arising from the NO story to date.

5.1.1 Therapeutic Applications

Notwithstanding its rapid reaction with oxygen in air, NO gas has proved surprisingly successful in the treatment of a number of conditions where selective vasodilatation in the pulmonary circulation is advantageous (Hayward et al. 2001). Principal amongst its uses are in pulmonary hypertension following surgery and in persistent pulmonary hypertension in the newborn. In both situations, NO is delivered in a mixture with nitrogen (N_2) in order to minimise its interaction with oxygen. Despite this precaution, higher oxides of nitrogen (NOx, some of which are highly toxic) have been shown to increase linearly with NO concentration, whilst the beneficial effects are seen to tail off. Inhaled NO should always be used under close supervision and the concentration should not exceed $\sim$80 ppm; concentrations of 100 ppb – 20 ppm are usually adequate to generate an optimal pulmonary response.

The therapeutic potential of inhaled NO is unlikely to extend beyond acute pulmonary conditions that can be closely regulated under medical supervision. Nevertheless, it has proved to be a life-saver in the particular clinical situations outlined above.

5.2 NO Donor Drugs

NO has unwittingly been used in the form of organic nitrates for over a century to treat angina; the discovery of NO as an endogenous messenger simply served to clarify the mechanism by which these ancient drugs worked. Since the 1980s, however, there has been a rapid proliferation in novel NO donor drugs, most of which have been developed with a therapeutic target in mind. Disappointingly, however, most have not made it to the clinical arena and instead are used as exploratory tools in the absence of any clear therapeutic advances. In this chapter, we explore the known mechanisms by which established and novel NO donating agents exert their effects and give indications as to what might be considered “best-practice” with respect to use of the agents in both pre-clinical and clinical studies.

5.2.1 Organic Nitrates

The organic nitrates are the best know class of NO donor, not only because they are one of only two NO donor classes in routine clinical use, but also due to the vast amount of research generated from this class of compound. As mentioned above, glyceryl trinitrate (GTN) has been used for relief of anginal pain for over 100 years and even today it is unrivalled in its efficacy. Although other organic nitrates are also used for this purpose, this section refers largely to GTN as it is the best-characterised organic nitrate and provides a good template for the group.

Structure and Activation

GTN contains three nitroxy ester groups, only one of which takes part in biologically relevant processes ("bioactivation"). Chemically, release of NO from a nitrate group requires a three electron reduction; although reducing agents (e.g. thiols) are undoubtedly involved in bioactivation, it is now almost universally assumed that specific enzymes mediate this process *in vivo* (Bennett et al. 1994). The identity of the nitrate-activating enzyme(s) has been intensely studied for over 40 years, generating a host of candidates and a mountain of confusing and often contradictory data. Early work focused on the ability of glutathione-S-transferases (GSTs) to release nitrite from GTN. Although largely discounted, this pathway highlights an important observation because, although GSTs can release nitrite from GTN, this does not appear to lead to bioactivation. The pharmacological actions of GTN are specifically associated with release of a terminal nitrate group (GSTs act on the middle nitrate group (Fig. 5) and measurement of the metabolite 1,2-glyceryl-dinitrate by thin layer chromatography and liquid scintillation is now used frequently to confirm biological responses. Cytochrome P450 enzymes and an unidentified membrane-bound enzymes have also been suggested (Servent et al. 1989; Chung and Fuung 1990), although attention has waned from these proposals.

Mitochondrial aldehyde dehydrogenase (mtALDH or ALDH-2), has recently arisen as a plausible candidate (Chen et al. 2002) and has now been shown to meet most criteria biological activation of GTN: it is an intracellular enzyme; it contains active sulfydryl groups that are redox regulated; it generates the correct metabolites and it releases NO from physiologically active concentrations of GTN. Further support for this candidate has come from mtALDH knockout mice or pharmacological inhibition of mtALDH (using chloral hydrate or benomyl), both of which show a marked attenuation of GTN activity but not other NO donors (Chen et al. 2002; Sydow et al. 2004). Desensitisation of mtALDH in nitrate tolerance (see below) has also been demonstrated, although this alone may not be able to account for all the effects seen in tolerance *in vivo* (De La Lande et al. 2004; Di Fabio et al. 2003). Further work is now required to look at the distribution of mtALDH in different blood vessels/vascular beds/other organs, the role of specific thiols in mtALDH regulation (Ignarro 2002) and determine why mtALDH only seems to bioactivate organic nitrates with 3 or more nitroxy ester groups (Wenzel et al. 2007).

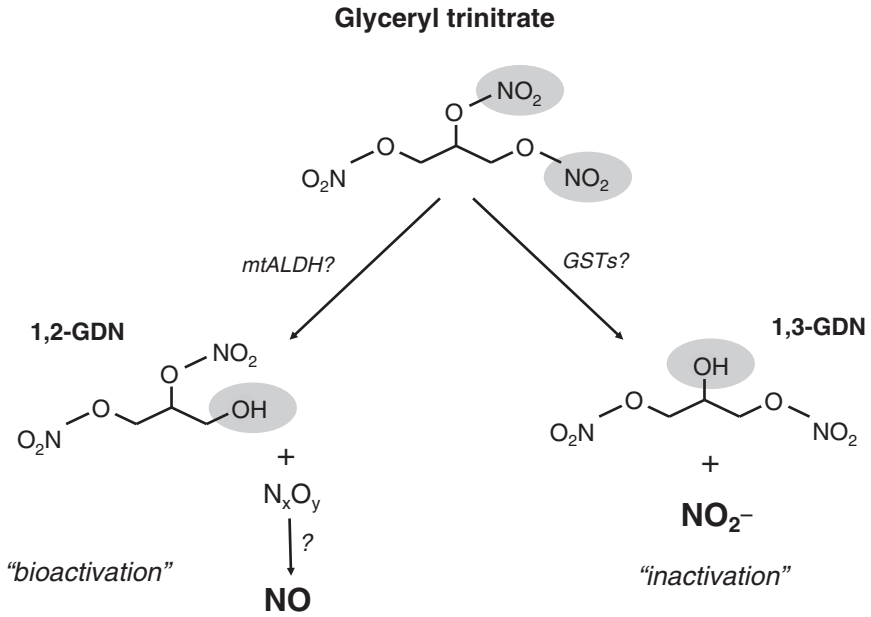


Fig. 5 Metabolic pathways for bioactivation and inactivation of glyceryl trinitrate

Nitrate Tolerance

The main limitation of organic nitrates is the loss of their effect with prolonged continuous use, so-called 'nitrate tolerance'. Currently, tolerance is avoided by introducing a nitrate-free interval in the therapeutic regimen. In general, this is a simple and effective solution, but it can be problematic for some forms of angina and limits the use of nitrates to the management of acute conditions. The subject of nitrate tolerance could easily fill a volume alone, therefore, here we cover only a few salient points (see Gori and Parker 2002a; Gori and Parker 2002b for succinct reviews of the literature). Early hypotheses of the causes of nitrate tolerance suggested that prolonged use of nitrates depleted tissue thiols key to the bioactivation. While it is generally accepted that this alone cannot account for all facets of the phenomenon, thiol depletion/oxidation is frequently observed and will likely influence many other cellular functions. Another early theory that has resurfaced recently is that prolonged nitrate use causes desensitization of sGC or increased breakdown of its key product cGMP by phosphodiesterases (Axelsson and Andersson 1983; Kim et al. 2001). The weakness of this theory is that other NO donors with less strict metabolic requirements still remain fully active in nitrate-tolerant tissues.

Two other theories of tolerance have clinical and experimental repercussions on the use of these drugs. Induction of tolerance *in vivo* is associated with increased generation of $^{\bullet}O_2^{-}$, proposed to scavenge GTN-derived NO (Munzel et al. 1995). The source of these radicals has been attributed to endothelial NAD(P)H-oxidases

and an uncoupling of eNOS, suggesting that nitrates may themselves cause endothelial dysfunction and contribute to cardiovascular disease progression. More recently, continuous use of high dose nitrates has been shown to downregulate mtALDH (Chen et al. 2002), raising concerns that nitrates may alter cell respiration and energy provision. Therefore, while tolerance may seem a relatively easy problem to overcome (by a nitrate-free interval or using an alternative NO donor), the problem of tolerance highlights a number of 'hidden' actions that nitrates may have on other important cellular functions that should be considered, especially in regards to the use of NO-hybrid drugs incorporating nitrate groups, such as nitro-aspirins.

Pentaerithrityl tetranitrate (PETN) deserves special consideration amongst organic nitrates. It contains four nitrate groups and accordingly is active at low concentrations (0.1–1 nM) but PETN does not appear to induce tolerance and remains active in GTN-tolerant vessels (Wenzel et al. 2007; Mollnau et al. 2006). Similarly, PETN also seems to cause less oxidative stress than other nitrates; indeed, there is a suggestion that PETN may also have a mild antioxidant effect (Kojda et al. 1998).

Therapeutics

GTN is frequently used for acute relief of pain associated with angina, while other slower release oral preparations, such as isosorbide mononitrate (ISMN), are used for the treatment of chronic angina and, occasionally, heart failure. The chemical properties of many organic nitrates, such as their lipophilicity has allowed them to be adapted into a number of preparations including ointments, transdermal patches and nebulisers, leading to alternative uses in treatment of anal fissure and pulmonary hypertension.

No other drug has rivalled GTN in the treatment of acute angina, yet the underlying reason for its effectiveness has yet to be fully elucidated. The most likely explanation is preferential activity of the organic nitrates for specific types of blood vessels. The organic nitrates are considered to preferentially dilate veins over arteries (although studies with isolated vessels do not support this assumption; Miller et al. 2008), acting to decrease preload to the heart without dramatically lowering blood pressure. Furthermore, they also exhibit a preference for large arteries over small, especially in the coronary circulation, thereby circumnavigating the drawback of coronary steal (redistribution of blood away from ischemic areas of the heart). A link between the chemical activation of nitrates and this profile of action has not yet been established, perhaps because the confounding reflex and regulatory responses of *in vivo* experiments, obscuring the true nature of these drugs in specific blood vessels and vascular beds.

Although GTN will continue to have a place in symptomatic relief for angina sufferers, a wider application for this class of NO donors seems unlikely. In addition to the tolerance-related effects of the organic nitrates, these drugs are weak antiplatelet agents *in vitro*, most likely because platelets lack the ability to bioactivate them. Both these factors may contribute to studies showing that the organic nitrates do not improve the outcome of patients with ischemic heart disease.

Practical Considerations

In most conduit arteries, GTN has an active concentration range (1 nM–10 μM) similar to that of other classes of NO donor; the activity of other organic nitrates varies with the number of nitrate groups in their structure (isosorbide dinitrate has a threshold concentration of 100 nM and isosorbide mononitrate at 1 μM). Studies with less potent nitrates are limited by their lipophilicity, in that it is difficult to make stock solutions above 10 mM without risking toxic effects of solvents in the final dilutions. Despite recognition as NO donors, the nitrates are relatively resistant to extracellular NO scavengers (e.g. hemoglobin and $^{\bullet}O_2^-$ generators). This likely reflects the intracellular generation of NO at a site close to sGC, although there are suggestions that GTN may act without the release of free NO at all (Kleschyov et al. 2003). Certainly, successful efforts to measure NO from nitrates have required supra-physiological concentrations of both nitrates and activators, bringing into question their relevance to the *in vivo* situation. In interpreting results from *in vitro* experiments, it is worth bearing in mind that the effects seen do not necessarily reflect the *in vivo* situation because of the lack of counter-regulatory mechanisms, the simplistic nature of the models and the acute treatment regimens.

5.2.2 Sodium Nitroprusside and Other Iron Nitrosyls

Sodium nitroprusside (SNP) is a well-known NO donating agent that has been used for almost a century as a powerful vasodilator, primarily in hypertensive crises during surgical operations, for controlled hypotension during anaesthesia and, very occasionally, in heart failure. Principal amongst the features that make SNP a useful agent in this setting is its very rapid and profound effects. However, these very features also underpin its major drawback in clinical practice: it is difficult to titrate on account of its steep dose-response effect and reversal in the event of overdose is also problematic. Furthermore, there are other practical limitations for the use of SNP: firstly, it contains 5 cyanide moieties and, despite their being tightly bound to an iron center, there have been isolated instances of cyanide poisoning with high dose or long-term treatment; secondly, SNP is light-sensitive and therefore requires careful storage and protection from light during infusion; and thirdly, SNP is not orally available, limiting its use to hospital-orientated applications, where it is delivered by intravenous infusion (0.3 μg/kg/min initially and gradually increasing to 6 μg/kg/min as necessary).

The mechanism by which SNP releases NO in tissue is not straightforward. Spontaneous release is unlikely in the absence of light and, indeed, NO has been found to be completely inactive in the dark in amphibian ventricular tissue. The same is not true in mammalian tissue, where a combination of thiol reduction and a tissue-specific protein are apparently responsible for the release process (Butler and Megson 2002).

In Clinical Studies

A major advantage for the use of SNP in clinical studies is that clinical-grade drug is available cheaply. As a result, the contra-indications and possible side-effects are well known and can be controlled for. Doses used in clinical studies are typically low and studies rarely extend for more than a few hours, avoiding the potential risk of cyanide poisoning. In addition, its extensive use for many years in this setting means that it is recognised to be the gold-standard for clinical studies and is generally accepted as the NO donor of choice.

The most extensive use of SNP in clinical studies in recent times is in support of studies investigating endothelial dysfunction. SNP is the endothelium-independent NO donor of choice to run alongside endothelium-dependent vasodilators such as acetylcholine, bradykinin and substance P. The strength of including SNP in studies involving endothelial dysfunction is that a depression in responses to endothelium-dependent relaxation might be due to endothelial dysfunction but could also be due to downregulation of the downstream activation pathway (sGC); using an NO donor like SNP facilitates discrimination between endothelial dysfunction and downstream dysfunction.

In Pre-Clinical Studies

SNP has largely been replaced in pre-clinical studies by other NO donors with more clearly defined NO release profiles. However, SNP is still often preferred if parallels with clinical studies are desired. The EC_{50} for SNP is typically $\sim$10–100 nM, and relaxation is usually maximal at $\sim$1–10 μM, depending on the tissue used. A consideration when selecting SNP is the recognition that relaxation mediated by this agent is less sensitive to attenuation by oxidative stress, presumably because of the close proximity of NO release to sGC. This can be seen as an asset because it confirms that downstream pathways are intact, but perhaps makes interpretation problematic, given that NO-mediated effects might be expected to be inhibited by oxidative stress, irrespective of the source.

Iron-sulfur nitrosyl clusters are a group of compounds that can be loosely associated with SNP on account of the Fe-NO moiety (Butler and Megson 2002). Compounds in this class are exemplified by Roussin's Black Salt, a high capacity NO donor containing 7 molar equivalents of NO. Iron-sulfur nitrosyls are less stable than SNP and undergo spontaneous thermal decomposition to yield NO, Fe_2O_3 and elemental sulfur. However, like SNP, decomposition is accelerated by exposure to light. Given the potentially toxic nature of the by-products generated from these compounds, it is hard to see any therapeutic use for them, except perhaps as photo-activated anti-tumor agents. They do, however, posses an interesting affinity for the endothelium of blood vessels, where they can reside for long periods, generating sustained vasodilator responses (Flitney et al. 1992).

5.2.3 Diazeniumdiolates (NONOates)

NONOates were first described in 1960 (Drago and Paulik 1960), but only since the discovery of NO as EDRF have they received attention in a biological setting. As expected, NONOates show most of the attributes of NO itself, including vasodilatation, inhibition of platelet aggregation and inhibition of vascular smooth muscle cell proliferation.

Structure and Activation

These compounds consist of a diolate group [N(O-)N = O] bound to a nucleophile adduct (a primary or secondary amine or polyamine) via a nitrogen atom (Maragos et al. 1991). NONOates decompose spontaneously in solution at physiological pH and temperature, to generate up to 2 molar equivalents of NO. NO release follows simple first order kinetics the rate of NO release can be accurately predicted from the half life and stoichiometry of NO release (assuming there are no extreme changes in temperature, pH and oxygen saturation of the solution or NO scavengers present; Schmidt et al. 1997). The rate of decomposition is dependent on the structure of the nucleophile. A range of NONOates has now been described with half-lives varying from seconds to hours (Fig. 6). An attractive feature of this class of compounds is that their decomposition is not catalysed by thiols or biological tissue, and therefore,

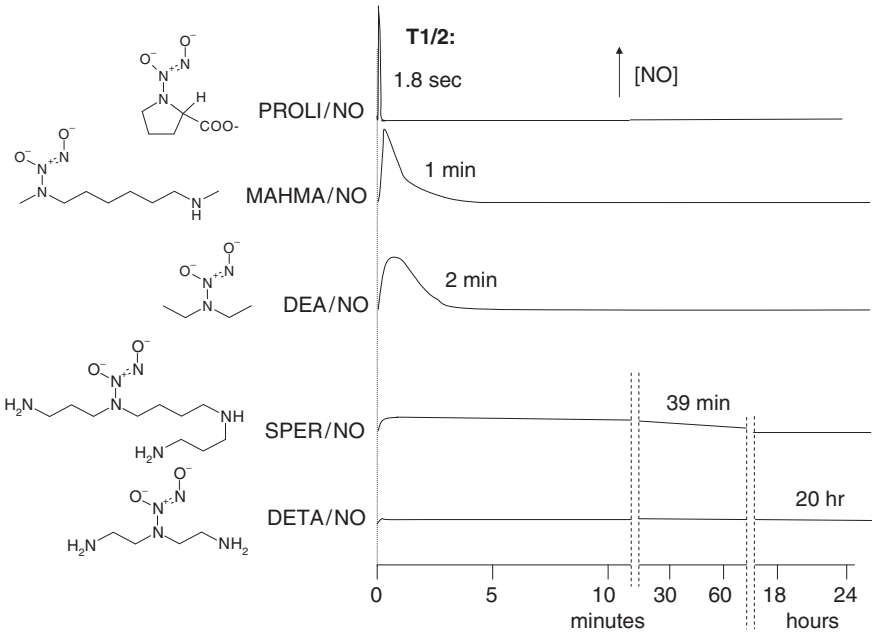


Fig. 6 Structures and NO release profiles for a range of NONOate compounds

biological activity closely correlates with the amount of NO generated *in vitro*. The lack of requirement for tissue to mediate NO release is likely to be responsible for the lack of tolerance seen with these compounds.

Therapeutic Potential

Currently, the NONOates have been used in only a few human studies and, subsequently, are unlikely to be used clinically in the near future. However, the predictable nature of NO release from NONOates makes them attractive compounds in numerous clinical applications, and their use in animal models of cardiovascular disease has been heavily investigated (see Keefer 2007 for review). NONOates have been investigated in many experimental models of cardiovascular disease and several NONOates have shown promising results in the prevention of thrombosis and neointimal formation following vascular injury by balloon angioplasty, endarterectomy, bypass grafting or placement of stents. Several rapidly acting NONOates have also shown benefits in a model of subarachnoid hemorrhage and pulmonary hypertension.

A great deal of research has been carried out to incorporate NO donors into gels and polymers for medical applications, such as interventional cardiology devices. This is especially true of NONOates, which have been successfully incorporated into stents, artificial grafts and perivascular gels, all of which have been shown to reduce platelet adhesion or restenosis. NONOates have also been used to coat the external surface of electrodes that measure blood gases and electrolytes, as thrombus formation on the sensor can interfere with measurements. The local effects of NO in this setting ensure that there is no impact on systemic blood pressure. As with other drug-eluting stents/grafts, long-term effectiveness will be the limitation of this approach because the NO in the coating is finite and will, at best, only last a few days.

Recent interest has centred on chemical alterations to the nucleophile adduct to protect the terminal oxygen of the diolate moiety, stabilising the drug in solution and potentially engendering selectivity for different organs, vascular beds or specific cell types (see Miller and Megson 2007). If vascular effects can be localised, NONOates may also have a use in the treatment of erectile dysfunction by enhancing blood flow to the penis, although it remains to be determined whether NONOates would have advantages over phosphodiesterase V inhibitors in this regard. Development of these ‘conjugated NONOates’ has expanded the potential of the diazeniumdiolates to include treatment of inflammatory conditions, leukemia and cancer. The potential for oral preparations of NONOates has yet to be fully clarified, although transdermal preparations have already been developed. Before clinical applications can progress, the toxicity of these compounds needs to be established, especially as subsequent reactions between decomposition products could lead to the formation of carcinogenic nitrosamines.

Practical Considerations

While the clinical future of NONOates remains uncertain, experimentally, the NONOates remain an invaluable scientific tool. It is becoming apparent that the release of NO from NO donors is highly complex, involving both chemical and biological derived process and nitrogen oxide species other than/as well as free NO. Other than the use of NO gas, NONOates are currently the only NO donors that specifically release NO in solution without interference from tissue factors. That said, several practical concerns should still be addressed. During handling of NONOates, the NO group must be protected to prevent premature release. This is normally done through preparation and dilution in alkaline solutions (e.g. 0.01 M NaOH), which is relatively straightforward, although care should be taken to make sure biological responses are due to NO and not the alkaline vehicle. Effective concentration range will depend on the half-life of NO release, but also the drug contact time. Therefore, potency may be very different in incubation conditions such as organ bath studies, compared to bolus administration to a perfused set-up, and analysis should consider the time course of the response (e.g. area under the curve rather than peak response). The most commonly used NONOates are DEA/NO and SPER/NO, which have active concentration ranges of 1 nM–10 μM and 10 nM–100 μM, respectively, in platelet rich plasma or standard organ bath preparations.

5.2.4 S-Nitrosothiols

Structure and Activity

The S-nitrosothiols are characterised by the RS-NO moity formed by the nitrosation of sulfhydryl (thiol) groups (Williams). Endogenously, S-nitrosothiols are found in small amounts as S-nitroso-cysteine and S-nitroso-glutathione (GSNO) as well as circulating in the blood as S-nitroso-albumin and S-nitroso-hemoglobin. S-Nitrosothiols may act as a short-term 'storage' for NO activity (Al-Sa'doni and Ferro 2000). Their formation can also be mediated by the reaction of low molecular weight thiols with peroxynitrite, although peroxynitrite is a relatively weak nitrosating agent and is more likely to oxidise thiol groups instead (Wink et al. 1994; Mayer et al. 1998).

Biological activity of S-nitrosothiols is highly influenced by the molecular environment of the parent thiol, especially in terms of stability and speed of release of NO. Many factors have been shown to induce the release of NO from S-nitrosothiols, including light, heat, transition metals, thiols, superoxide and enzymes such as xanthine oxidase, superoxide dismutase, protein disulphide isomerase and various dehydrogenases (Al-Sa'doni and Ferro 2000). Subsequently, S-nitrosothiols have advantages over other classes of NO donors, such as the organic nitrates, since they have far less stringent metabolic requirements and this may be the reason that they do not induce tolerance with long-term use.

The complex chemistry of the S-nitroso bond also allows these compounds several means by which they can confer NO bioactivity. In particular, S-nitrosothiols may directly donate bioactivity through transfer of nitrosonium ions (NO^+) rather than free NO. In disease settings, this may be advantageous in that higher oxides of nitrogen activity is protected from scavenging agents such as oxygen-centred free radicals. This mechanism could also allow large molecule weight S-nitrosothiols to transfer oxides of nitrogen across cell membranes to subcellular targets (Stamler et al. 1992; Zai et al. 1999). S-Nitrosothiols have been shown to exert biological effects that are independent of sGC, including cGMP-independent vasodilatation and anti-platelet effects, alterations in enzyme function and regulation of ion channels and receptors. It is likely that transfer of direct NO^+ may account for several of these actions, especially those of enzymes with sulfydryl groups in their active sites (Stamler 1995). It should be noted that other NO donors, such as the NONOates, also exhibit cGMP-independent effects at concentrations that generate high levels of extracellular NO, allowing reaction with molecular oxygen to form nitrosating species such as N_2O_3 or NO_2.

Therapeutic Potential

S-Nitrosothiols have a number of potential advantages over other classes of NO donors. Firstly, some examples show tissue selectivity: GSNO is selective for arteries over veins, giving them a different haemodynamic profile of action that those of classical organic nitrates (MacAllister et al. 1995). Additionally, S-nitrosothiols are potent antiplatelet agents, inhibiting aggregation at doses that do not influence vascular tone (De Belder et al. 1994). The endogenous nature of S-nitrosothiols and in vivo tolerability to even high concentrations suggest that, with careful design, most S-nitrosothiols are unlikely to be cytotoxic at pharmacologically relevant concentrations.

S-Nitrosothiols are not used clinically at present, but several clinical studies demonstrate their advantageous features, especially in the cardiovascular system (see Miller and Megson 2007). GSNO has been shown to decrease the occurrence of cerebral embolism after carotid endarterectomy in patients already receiving aspirin and heparin. Additionally, GSNO inhibits platelet adhesion in bypass grafts and thrombosis following coronary angioplasty or emboli that dissociate from carotid plaques. Numerous animal models have demonstrated further clinical potential of S-nitrosothiols in cardiovascular disease, including atherosclerosis and myocardial ischemia. S-nitroso-albumin reduces platelet adhesion and neointimal thickening in angioplasty-damaged blood vessels, when given as an infusion or as a stent-coating. In addition, several novel lipophilic S-nitrosothiols (Fig. 7) are selectively retained in the lipophilic areas at sites of endothelial damage, where they slowly release NO over a prolonged period (Megson 2002; Megson and Webb 2002; Miller et al. 2003).

Outside the cardiovascular system, GSNO has been shown to have neuroprotective properties and to minimise liver deterioration during transplant surgery. GSNO

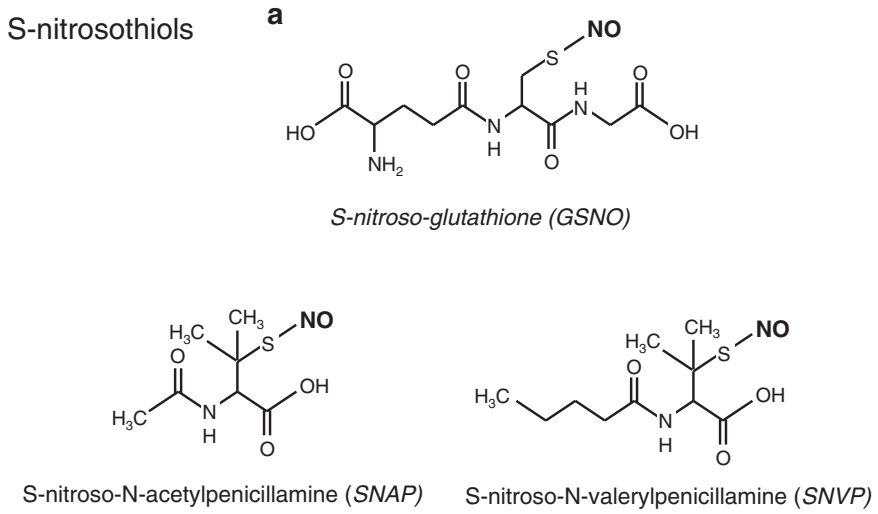


Fig. 7 Chemical structures of some important S-nitrosothiols

also encourages collagen deposition during healing of cutaneous incisions and therefore may show benefits for the development of wound dressings, particularly once delivery has been optimised e.g. by structural modifications to improve stability or lipophilicity. Nitrosation of the thiol groups of existing and modified drugs has created a wide range of S-nitroso-hybrid drugs for the treatment of cardiovascular disease, inflammation and medical devices (covered below).

Practical Considerations

Similarly to SNP, S-nitrosothiols need to be protected from light during handling to prevent premature release of NO. Transition metal ions such as copper also initiate decomposition. This can be problematic because all physiological buffers will contain trace amounts of these metals, therefore, particularly susceptible S-nitrosothiols such as S-nitroso-cysteine need to be prepared immediately before use. Synthesis of S-nitrosothiols is also difficult as the product with likely contain an amount of unreacted thiols, which initiate a chemical cycle by reducing trace metals to a form that catalyses decomposition (e.g. Cu^{2+} to Cu^{+}). A number of synthetic thiols are now available which have greater stability due to steric hindrance of the S-NO bond preventing metal-mediated catalysis. However, in some cases the low yields and the lack of purity of novel product may preclude the agent reaching clinical trials. Nevertheless, the endogenous S-nitrosothiol, GSNO, is potentially inexpensiveand likely to be non-toxic. Working concentrations are generally between 1 nM–10 μM for NO-mediated effects such as platelet inhibition and vasodilatation, depending on the tissue used and co-factors available. GSNO is not retained within tissue, but

other S-nitrosothiols such as S-nitroso-albumin or lipophilic agents, may accumulate at certain cellular sites, therefore temporal effects of high doses of these agents should be considered.

5.2.5 Peroxynitrite Generators (Sydnonimines and Mesoionic 3-Aryl-Substituted Oxatriazole-5-Amines)

Originally regarded to be NO donors, is now clear that molsidomine, its active metabolite 3-morpholinosydnonimine (SIN-1; both sydnonimines; Fig. 8) and a class of compounds known as the mesoionic 3-aryl-substituted oxatriazole-5-amine derivatives (e.g. GEA3162; Fig. 8) are better described as peroxynitrite ($ONOO^-$) donors on account of their ability to co-generate NO and $^{\bullet}O_2^-$ in similar proportions, which rapidly react to form $ONOO^-$. The situation in the cellular environment, however, is more complex, with the high levels of antioxidants present in cells quenching a high proportion of the oxygen-centred radicals and unmasking the NO (Schrammel et al. 1998). Nevertheless, GEA-3162, for example, has a significantly higher pro-apoptotic effect than a 'pure' NO donor drug, which has been suggested to be due to formation of $ONOO^-$ (Taylor et al. 2004). In light of these findings, it is important that we re-assess the earlier findings with these compounds – their pharmacological properties might well be better explained in terms of $ONOO^-$, NO or a combination thereof. Whilst $ONOO^-$ is a weak activator of sGC, it is generally accepted that many of the actions of sydnonimines and related compounds are mediated via both cGMP-dependent (Weber et al. 1993) and cGMP-independent pathways including activation of K^+ channels (Plane et al. 1996).

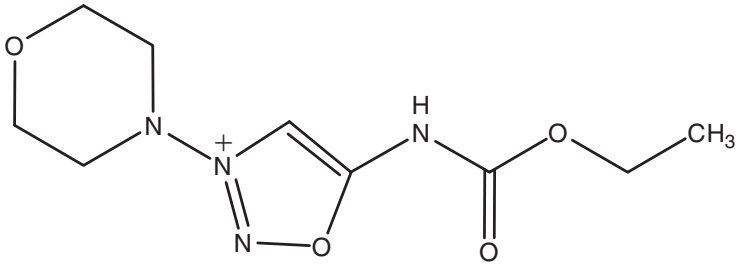
Molsidomine is the metabolic precursor of 3-morpholinosydnonimine (SIN-1). Conversion of molsidomine to SIN-1 occurs primarily in the liver but release of NO from SIN-1 involves a 2-step process that occurs spontaneously in the blood.

In Clinical Studies

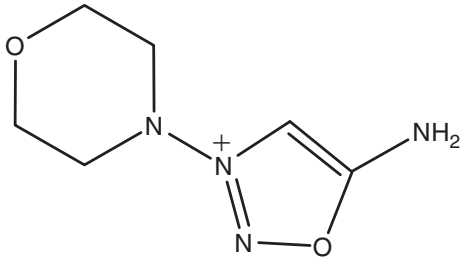
Molsidomine and SIN-1 are vasodilators that received some attention in the early 1980s as possible tolerance-free alternatives to organic nitrates in a range of cardiovascular conditions. However, they have largely failed to live up to expectation in this setting.

In Pre-Clinical Studies

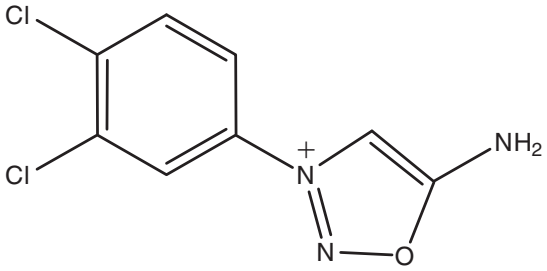
SIN-1 has been shown to protect against reperfusion injury-induced thrombosis, although claims that it might also help to prevent atherosclerosis by inhibiting the oxidation of low-density lipoprotein and smooth muscle cell proliferation are disputed, particularly given that $ONOO^-$ is pro-oxidant.



Molsidomine



SIN-1



GEA-3162

Fig. 8 Chemical structures of peroxynitrite-generating compounds

Mesoionic 3-aryl-substituted oxatriazole-5-amine derivatives display a full range of NO-related actions including vasodilatation, inhibition of platelet aggregation, antibacterial activity, modulation of neutrophil function and induction of neutrophil apoptosis (Taylor et al. 2003) but their benefits have yet to be confirmed in clinical studies.

5.2.6 NO Hybrids

One aspect of NO donor development over the past few years which might have a real impact on clinical practice is generation of hybrid NO donor drugs, that is to say NO-generating analogues of often well known drugs (Turnbull et al. 2006). There are two major concepts underpinning hybrid development:

- to overcome the side-effects of a drug by delivery of NO (e.g. gastro-toxicity of aspirin and other non-steroidal anti-inflammatory drugs; NSAIDs)
- to add to or synergise with the effects of conventional therapies (e.g. NO adducts of statins)

The potential benefits of linking NO-containing moieties to NSAIDs is exemplified by NCX-4016 (Fig. 9; Del Soldato et al. 1999). Addition of an organic nitrate moiety via a linker to aspirin does not appear to affect the anti-thrombotic and anti-inflammatory properties of the parent drug, but instills the added benefit of protection against ulceration of the gastric mucosa. NCX-4016 also reduces restenosis after arterial injury in mice, primarily through reduced vascular smooth muscle cell proliferation and macrophage accumulation at the site of injury, as well as preventing thromboembolism and ischemia-induced focal brain damage in rats. S-Nitroso-ester and nitrate ester derivatives of other NSAIDs, including, diclofenac (Fig. 9) have similar gastric mucosa-sparing properties to NCX-4016 and trials to test the clinical potential of NO/NSAID hybrids in the cardiovascular arena are ongoing.

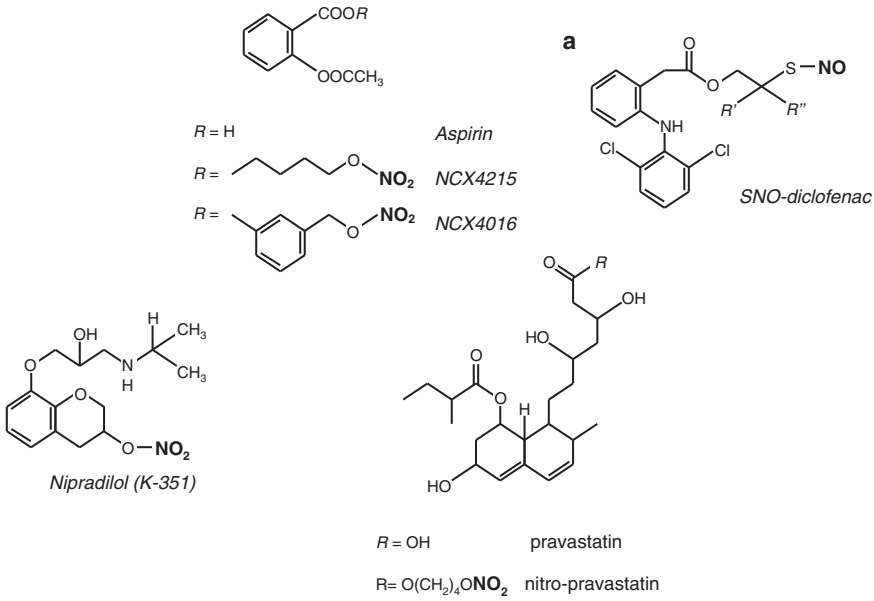


Fig. 9 Chemical structures for some important NO hybrid drugs

The implications are that these compounds might be of benefit in a vast range of disorders including inflammation, pain, gastro-intestinal disorders and asthma.

A range of other NO donor hybrid drugs has since developed, including nitro-oxylated/nitrosated histamine (H_2) receptor antagonists and nitrated cycloxygenase-2 (COX-2) inhibitors. In both cases, it is claimed that the NO-hybrid drugs have additional benefits over the parent compounds. Such hybrids might have a range of applications in inflammatory conditions, including renal disorders. This might prove a potential hindrance to use of NO-COX-2 compounds. Nipradilol is a β-blocker with NO-donating properties that has been shown to possess unique anti-atherogenic effects that could give it an edge over other anti-hypertensive agents in a very congested drug market.

A range of S-nitrosated proteins has also been generated (see Megson and Webb 2002 for review), including the endogenous fibrinolytic agent, tissue plasminogen activator (tPA) that is often administered to patients with myocardial infarction in an effort to disrupt blood clots in coronary arteries; S-nitrosated tPA apparently has the additional benefit of cGMP-dependent vasodilatation and inhibition of platelet aggregation over native tPA. Similarly, nitrosation of a recombinant fragment of the endogenous inhibitor of platelet adhesion, von Willebrand factor, enhances its ability to prevent platelet aggregation and adhesion. A major benefit of such an approach is that all the components of the drugs are endogenous and are unlikely to have toxic effects at biologically active concentrations.

5.2.7 NO Donating Materials

NO Hydrogels generating hydrogels comprise a range of NO donor-containing biocompatible macromers. Polyethylene glycol-based gels have been co-polymerised with S-nitrosocysteine and complexes of NO with poly-L-lysine and DETA nucleophiles have also been synthesised. Early studies suggest that they have anti-restenotic and anti-thrombotic properties, which might be useful in the context of stent coatings. Examples of these materials have also been shown to inhibit proliferation of cultured smooth muscle cells and to prevent platelet adhesion to collagen-coated glass slides. Out with the cardiovascular system, NO hydrogels might be an effective means of delivering NO to skin wounds, where it has previously been shown to help prevent infection and to accelerate healing.

Zeolites

A recent novel approach to storage and delivery of NO has recently been adopted using ion-exchanged zeolites (Wheatley et al. 2006). These microporous insoluble materials form a framework containing metal ions that can bind NO. Exposure of the solid to NO gas results in NO binding to the metal ions within the pores, facilitating highly efficient packing of NO within the solid; NO is displaced by water on immersion in an aqueous environment. These materials constitute very high capacity

stores for NO and the rate of release can be modulated by altering the porosity of zeolite, the metal ion in the framework and the composition and nature of the binder (Wheatley et al. 2006). This infinite flexibility with respect to NO release allows for development of a range of different NO donor materials for different purposes ranging from antimicrobial coatings for urinary catheters and wound dressings to durable, slow release anti-thrombotic coatings for stents, bypass tubing, cannulae and catheters. This approach represents a novel means of site-selective delivery of NO that optimises the benefits of NO as a local mediator.

6 Conclusion

NO is a crucial signalling molecule in a range of systems throughout the body, promising development of a number of important breakthroughs with respect to novel NO donating drugs. That the promise has not yet been realised might lie in the lack of specificity of the majority of NO donor drugs generated to date: the multiplicity of the roles for NO is likely to be a hindrance to systemic drug delivery leading to off-target effects. Design of novel drugs for specific therapeutic targets should consider new means of targeted delivery that might prevent non-specific and undesirable side effects.

NO donor drugs have proved an invaluable tool for exploring the mechanism of action of NO in a range of systems. It is worth re-iterating that selection of the appropriate NO donor for a particular experiment is critical in determining the validity of the experiment and the interpretation derived from the results. There is no 'typical' NO donor – each has its own specific characteristics that will determine the rate of release, peak concentration and NO-related species that is generated – a thorough understanding of the profiles of the various NO donor classes is a useful asset in selecting the correct donor(s) for a particular application.

A clear understanding of the intricacies of NO activity and drug characteristics is likely to lead to more selective agents that could penetrate therapeutic markets.

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NO-Independent, Haem-Dependent Soluble Guanylate Cyclase Stimulators

Johannes-Peter Stasch and Adrian J. Hobbs

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Abstract The nitric oxide (NO) signalling pathway is altered in cardiovascular diseases, including systemic and pulmonary hypertension, stroke, and atherosclerosis. The vasodilatory properties of NO have been exploited for over a century in cardiovascular disease, but NO donor drugs and inhaled NO are associated with significant shortcomings, including resistance to NO in some disease states, the development of tolerance during long-term treatment, and non-specific effects such as post-translational modification of proteins. The development of pharmacological agents capable of directly stimulating the NO receptor, soluble guanylate cyclase (sGC), is therefore highly desirable. The benzylindazole compound YC-1 was the first sGC stimulator to be identified; this compound formed a lead structure for the development of optimized sGC stimulators with improved potency and specificity for sGC, including CFM-1571, BAY 41-2272, BAY 41-8543, and BAY 63-2521. In contrast to the NO- and haem-independent sGC activators such as BAY

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58-2667, these compounds stimulate sGC activity independent of NO and also act in synergy with NO to produce anti-aggregatory, anti-proliferative, and vasodilatory effects. Recently, aryl-acrylamide compounds were identified independent of YC-1 as sGC stimulators; although structurally dissimilar to YC-1, they have a similar mode of action and promote smooth muscle relaxation. Pharmacological stimulators of sGC may be beneficial in the treatment of a range of diseases, including systemic and pulmonary hypertension, heart failure, atherosclerosis, erectile dysfunction, and renal fibrosis. An sGC stimulator, BAY 63-2521, is currently in clinical development as an oral therapy for patients with pulmonary hypertension. It has demonstrated efficacy in a proof-of-concept study, reducing pulmonary vascular resistance and increasing cardiac output from baseline. A full, phase 2 trial of BAY 63-2521 in pulmonary hypertension is underway.

1 Limitations of Nitric Oxide Pharmacotherapy in Cardiovascular Disease

Nitric oxide (NO) stimulates vasodilatation by triggering a signalling cascade beginning with the activation of soluble guanylate cyclase (sGC), which generates the second messenger, cyclic guanosine-3′,5′-monophosphate (cGMP). The increased level of cGMP modulates the activity of downstream effectors including protein kinases, phosphodiesterases (PDE), and ion channels, ultimately regulating vasodilatation and vascular smooth muscle cell growth.

The pharmacological properties of NO have been exploited for over a century in cardiovascular disease (Brunton 1867), and to this day, NO donor drugs remain an important feature of cardiovascular medicine (Murad 2006). However, a number of cardiovascular diseases such as ischaemic heart disease and hypertension are associated with resistance to NO donor drugs (Chirkov and Horowitz 2007), and a significant proportion of patients with pulmonary hypertension (PH) do not respond to inhaled NO (Klinger et al. 2006; Krasuski et al. 2000). NO resistance has equally important consequences for smooth muscle and platelet function, reducing vasodilatation, and increasing the propensity for platelet aggregation. Impaired responsiveness to NO in ischaemic patients implies that those patients in greatest need of NO donor therapy may be least likely to respond. Furthermore, both inhaled NO and NO donor drugs are associated with additional limitations including many non-specific (cGMP-independent) effects, and long-term use of NO donor drugs results in the development of nitrate tolerance.

A number of factors contribute to the development of NO resistance in cardiovascular disease and nitrate tolerance following long-term use of NO donor drugs, including insufficient NO release from NO donor drugs, hyper-contractile responses to vasoconstrictors (e.g., noradrenaline), and increased vascular superoxide production (Chen et al. 2002; Munzel et al. 2005). Mitochondrial aldehyde dehydrogenase (ALDH), the enzyme required for release of NO from organic nitrates (Chen et al. 2002; Munzel et al. 2005; Mayer and Beretta 2008), is thought to play a role in

nitrate tolerance; ALDH activity is impaired in nitrate-tolerant blood vessels (Chen et al. 2002), and a common variant of ALDH2 (Glu504Lys) has severely compromised function, which causes resistance to glyceryl trinitrate (Li et al. 2006). Administration of NO gas by inhalation, circumvents the requirement for functional ALDH, and achieves pulmonary vasodilatation without systemic vasodilatation in newborns with pulmonary hypertension and hypoxia (Bloch et al. 2007). However, inhaled NO has only short-term efficacy (Olschewski et al. 1996), and there is a risk of rebound hypertension following withdrawal of the therapy (Atz et al. 1996; Ichinose et al. 2004).

Oxidative stress contributes to the tolerance of inhaled NO and NO donor drugs. Cardiovascular diseases are often associated with elevated levels of angiotensin II, which stimulates NAD(P)H oxidase to increase production of superoxide (Harrison et al. 2003). Long-term treatment with NO also increases vascular superoxide production via PKC-mediated activation of NAD(P)H oxidase and endothelial NO synthase (NOS) uncoupling. The increased levels of superoxide promote the 'scavenging' of NO to form peroxynitrite (Munzel et al. 2005; Munzel et al. 2007) and may cause oxidation of the haem unit in sGC, rendering the enzyme unresponsive to NO (Gupte et al. 1999; Gladwin 2006; Stasch et al. 2006). Whether oxidative stress always correlates with the occurrence of oxidized/haem-free sGC remains to be proved; alternative mechanisms may exist, including deficient haem biosynthesis or incorporation (Chirkov and Horowitz, 2007; Mingone et al. 2008). Reduced expression of sGC has also been observed in cardiovascular disease models and may contribute to NO resistance (Melichar et al. 2004; Ruetten et al. 1999). In contrast, increased sGC expression has been observed in animals made tolerant to organic nitrates (likely as a compensatory mechanism), although downstream processes such as VASP phosphorylation remain suppressed (Mulsch et al. 2001). In addition, increased PDE-1 and PDE-5 activity has been proposed to play a role in nitrate tolerance (Kim et al. 2001; MacPherson et al. 2006; Pagani et al. 1993); this may also promote cross-tolerance with the natriuretic peptide system by reducing cGMP signalling downstream of the natriuretic peptide receptors, particulate guanylate cyclases.

Inhaled NO and NO donor drugs suffer from additional limitations. For example, NO has many non-specific effects, including tyrosine nitration (Amirmansour et al. 1999; Radi 2004), S-nitrosation (Handy and Loscalzo 2006), DNA damage (Burney et al. 1999; Nguyen et al. 1992), prevention of nucleotide excision repair (Chien et al. 2004), and interactions with metal-containing proteins in the mitochondria, for example cytochrome oxidase (Erusalimsky and Moncada 2007). Physiological levels of NO interact with cytochrome oxidase in mitochondria, inhibiting mitochondrial oxygen uptake, altering electrochemical gradients, causing the generation of reactive oxygen species and influencing processes such as the release of pro-apoptotic proteins. Peroxynitrite formed by supraphysiological levels of NO induces more persistent and severe mitochondrial oxidative damage via interaction with aconitase and members of the mitochondrial electron transport chain (Radi et al. 2002). Peroxynitrite may also contribute to oxidative stress by preventing oxidative DNA damage repair (Phoa and Epe 2002), and causing lipid peroxidation

(O'Donnell and Freeman 2001). Finally, studies of patients with acute coronary syndromes including myocardial infarction have failed to show any clear evidence for a positive impact of NO-based therapy on mortality (GISSI-3 1994; ISIS-4 Collaborative Group 1995; Thadani and Ripley 2007).

The beneficial actions of NO are mediated almost exclusively via activation of sGC (e.g., vasodilatation, and inhibition of smooth muscle proliferation, leukocyte recruitment and platelet aggregation), whereas the detrimental effects of NO are predominantly cGMP-independent (as described above). These limitations have therefore driven research into drugs that bypass NO and act directly on sGC to restore the sGC-cGMP signalling pathway in cardiovascular disease.

2 NO-Independent, Haem-Dependent Soluble Guanylate Cyclase Stimulators

2.1 The Indazole Family

Direct NO-independent sGC stimulation was first demonstrated in 1994, when Ko and colleagues reported a synthetic benzylindazole compound called YC-1, as a stimulator of cGMP synthesis (Ko et al. 1994), which was subsequently verified to be a direct sGC stimulator (Friebe et al. 1996; Mulsch et al. 1997). In 1994, scientists at Bayer AG (Wuppertal, Germany) screened 20,000 compounds for stimulators of cGMP synthesis, leading to the identification of 5-substituted-2-furaldehyde-hydrazone derivatives as direct sGC agonists with a similar structure to YC-1. However, the potency of these first compounds increased upon exposure to light, rendering them unsuitable for further drug development.

YC-1 formed a lead structure for the development of next-generation sGC activators such as CFM-1571, BAY 41-2272, BAY 41-8543, and finally BAY 63-2521 (Fig. 1). YC-1 and its successors stimulate sGC independently of NO, but they also act in synergy with NO. Moreover, they all depend on the presence of a reduced haem moiety within sGC. In contrast, sGC activators such as BAY 58-2667 mimic the haem group and activate sGC in an NO- and haem-independent manner (see Chap. "NO- and haem-independent soluble guanylate cyclase activators").

2.1.1 YC-1

The synthetic benzylindazole compound YC-1 was first documented in 1978 (Jackson et al. 2007; Yoshina et al. 1978), but its role in cGMP signalling was recognized only 16 years later, when it was presented as an anti-thrombotic agent that inhibits platelet aggregation by increasing platelet cGMP levels in an NO-independent manner (Ko et al. 1994). YC-1 was also found to act in synergy with NO (Hoenicka et al. 1999; Mulsch et al. 1997; Schmidt et al. 2001).

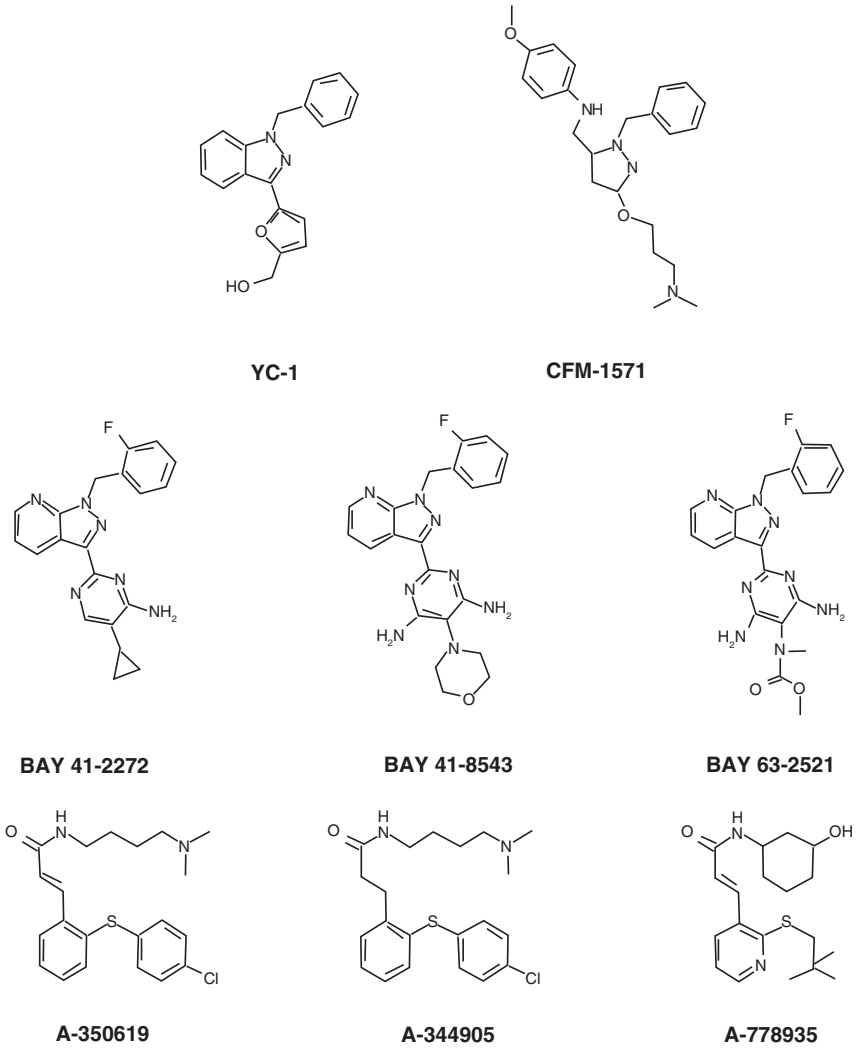

Fig. 1 Nitric oxide independent stimulators of soluble guanylate cyclase – indazole and aryl-acrylamide families

The ability of YC-1 to stimulate sGC depends upon the presence of a reduced haem moiety, which is able to bind NO or protoporphyrin-IX, the iron-free precursor of haem that structurally resembles the NO-haem complex (Friebe et al. 1996). The haem group is thought to associate with the β1 subunit of sGC as a five-coordinated ferrous haem with histidine-105 as the axial ligand (Stone and Marletta 1994). Interactions with a recently-identified haem-binding motif (Y-x-S-x-R) have also been described (Ma et al. 2007; Pellicena et al. 2004; Schmidt et al. 2005; Schmidt et al. 2004). Binding of NO to the sixth coordination position of the haem

causes scission of the histidine-iron bond and induces structural changes within the haem moiety which is thought to trigger the conformational change required to activate the enzyme.

The exact mechanism by which sGC enzyme activation occurs remains under debate. A two-step process has been proposed, in which the second step regulates the transition of NO-bound sGC from a low-output to a high-output activated state. The low-output form of sGC was shown to release bound NO slowly, whereas NO dissociation from the high-output form was believed to occur rapidly (Winger et al. 2007); in accord, YC-1 was recently shown to accelerate the rate of NO release from sGC, suggesting that YC-1 promotes formation of the high-output form of sGC (Winger et al. 2007). Recent studies have also proposed the involvement of a non-haem NO-binding site in the sGC activation process (Cary et al. 2005; Derbyshire and Marletta 2007; Derbyshire et al. 2008), and suggested a role for the reaction products of sGC (Russwurm and Koesling 2004). Although such data provide some support for the two-step hypothesis, the theory remains controversial, and recent experimental data support an alternative model that predicts a one-step process with a single binding site for NO, akin to classical agonist-receptor interactions (Roy and Garthwaite 2006).

YC-1 is thought to stabilize sGC in its active configuration by stabilizing the nitrosyl-haem complex (Friebe and Koesling 1998; Russwurm et al. 2002). A study by Makino and colleagues indicated that YC-1 weakens or cleaves the proximal histidine-iron bond, when NO or carbon monoxide (CO) is bound (Makino et al. 2003). CO alone is a poor activator of sGC, being approximately 80-fold less potent than NO; addition of YC-1 was found to increase the efficacy of CO dramatically, to a level similar to that observed with NO (Friebe et al. 1996; Hoenicka et al. 1999; Kharitonov et al. 1999; McLaughlin et al. 2000; Sharma et al. 1999; Stone and Marletta 1998). Thus, it is possible that low levels of an endogenous allosteric stimulator of sGC with similar properties to YC-1 may account for the biological activity of CO via sGC in vivo.

YC-1 causes an approximately 10-fold increase in purified sGC activity ($EC_{50} = 20\,\mu M$), largely by increasing the maximal catalytic rate, although a decrease in the K_M for the substrate GTP has also been observed. Synergy with NO increases the effect of YC-1 by approximately 1–2 orders of magnitude (Friebe et al. 1996; Lee et al. 2000; Mulsch et al. 1997).

The precise molecular mechanism by which YC-1 exerts its stimulatory effects on sGC remains unknown. Mutation studies imply an interaction between YC-1 and the catalytic domain of sGC, similar to forskolin-dependent activation of the closely related adenylate cyclase enzyme (Friebe et al. 1999, 1997; Lamothe et al. 2004; Tesmer et al. 1997). A photoaffinity-labelling approach was used to demonstrate labelling of cysteine 238 and cysteine 243 in the sGC α-subunit upon binding of BAY 41-2272, a compound based on YC-1 (Stasch et al. 2001). This result was supported by a study of photolabile derivatives of YC-1, which labelled the sGC α-subunit (Hering et al. 2006), and deletion studies in which a region of the sGC α-subunit encompassing amino acids 259–364 was implicated in transduction of the NO-binding signal and NO sensitization by YC-1 (Koglin and Behrends 2003).

However, as no crystal structure of the α/β sGC is yet available, residues identified by photoaffinity-labelling might be situated in the interface between both sGC subunits, thus allowing labelling of the sGC α-subunit, even if the compound binds to the corresponding β-subunit. This view is supported by subsequent mutational studies of the sGC α-subunit, which showed no effect (Schmidt et al. unpublished data). Furthermore, Denninger and colleagues demonstrated the interaction of YC-1 with fragments of sGC containing only the haem-binding domain of the β-subunit (Denninger et al. 2000). Certain residues in this region are thought to be responsible for the translation of the NO-binding signal into activation of the sGC enzyme (Pellicena et al. 2004), but their role in YC-1 mediated activation remains to be established. In summary, evidence to date has implicated both subunits of sGC in the action of YC-1, and interaction of YC-1 with the catalytic domain has been proposed. However, the precise mechanism by which YC-1 stimulates sGC remains to be established, and awaits verification by co-crystallization studies.

In pharmacological studies, YC-1 exhibited relatively weak (2–3 fold less potent than next-generation compounds) vasodilatory, anti-remodelling, and anti-platelet properties. For example, YC-1 stimulated relaxation in human radial and mesenteric artery rings, rat mesenteric artery rings, porcine pulmonary arteries, rabbit aortic rings and corpus cavernosum, and rat anococcygeus muscle, corpus cavernosum, and myometrium (Berkan et al. 2007; Cetin et al. 2004; Che et al. 2005; Demirkoprulu et al. 2005; Gonzalez-Luis et al. 2006; Hsieh et al. 2003; Mulsch et al. 1997; Nakane et al. 2002; Slupski et al. 2007; Teixeira et al. 2006b). Furthermore, YC-1 has been shown to sensitize nitrate-tolerant rabbit aortic rings to NO (O'Reilly et al. 2001). Proliferation of rat smooth muscle cells in culture is inhibited by YC-1 (Tulis et al. 2002), and vascular smooth muscle cell apoptosis, which plays a role in the formation of lesions in vascular disease, is also prevented by the compound (Pan et al. 2004). In addition, YC-1 stimulates Vasodilator-Stimulated Phosphoprotein (VASP) phosphorylation in platelets, inhibits platelet aggregation and adherence to collagen, and prolongs rat tail bleeding time (Becker et al. 2000; Friebe et al. 1998; Teng et al. 1997; Tulis et al. 2002; Wu et al. 1995).

A role for YC-1 in the enhancement of long-term potentiation and learning and memory has also been proposed (Bredt 2003; Chien et al. 2003, 2005; Garthwaite 2008). YC-1 enhanced the induction of long-term potentiation and the active shuttle avoidance response in rats (Chien et al. 2008). Immunofluorescence studies of rat brain coronal slices suggest that YC-1 activates cGMP synthesis in GABAergic and glutamatergic cells that do not respond to NO per se (van Staveren et al. 2005)

YC-1 is orally active, at least at high doses (Becker et al. 2000; Huang et al. 2005; Liu et al. 2006), and the compound has beneficial effects in animal models of cardiovascular disease, stimulating a pronounced decrease in mean arterial pressure in a rat model of hypertension (Rothermund et al. 2000), and reducing neointima formation following arterial balloon injury in rats (although Liu and colleagues suggested that this may occur independently of cGMP) (Liu et al. 2006; Tulis et al. 2000, 2002; Wu et al. 2004).

A major limitation of YC-1 as a stimulator of sGC is its lack of specificity. It was reported to inhibit PDEs (Friebe et al. 1998; Galle et al. 1999), and was shown to have

many cGMP-independent effects including the modulation of lipopolysaccharide-induced inflammatory responses, effects on ion channels, inhibition of proliferation, and stimulation of apoptosis. For example, YC-1 affects lipopolysaccharide-induced maturation of dendritic cells, inhibiting their capacity to activate allogenic T-cells (Tsai et al. 2007), and potentiates lipopolysaccharide/interferon gamma-induced tumour necrosis factor (TNF) release from a rat alveolar macrophage cell line (Hwang et al. 2003), both in a cGMP-independent fashion. YC-1 also inhibits tyrosine kinase activity and calcium influx in rat neutrophils in a cGMP-independent manner (Wang et al. 2001). Moreover, studies in human radial and mesenteric artery rings indicate possible cGMP-independent stimulation of calcium-sensitive potassium channels and sodium pumps, respectively (Berkan et al. 2007; Slupski et al. 2007). A relatively high concentration of YC-1 (30 μM) inhibits sodium channel activity in rat neurons independently of its interaction with sGC (Garthwaite et al. 2002), and the compound affects calcium-activated potassium currents in a rat lactotroph cell line, without increasing cGMP formation (Wu et al. 2000). Anti-proliferative cGMP-independent effects have also been observed in rat mesangial cells (Chiang et al. 2005), human hepatocellular carcinoma cells (Wang et al. 2005a), human umbilical vein endothelial cells (Hsu et al. 2003), and human vascular endothelial cells (Pan et al. 2005b).

The potential of YC-1 as an anti-cancer agent has generated much interest. YC-1 prevents tumour growth, invasion, and metastasis in immunodeficient mice (Shin et al. 2007; Yeo et al. 2003) and in vitro studies have demonstrated that YC-1 prevents tumour cell growth and induces apoptosis (Yeo et al. 2006; Zhao et al. 2007; Wu et al. 2008). Studies in hypoxic cancer cells have revealed that the effects of YC-1 are largely achieved through a reduction in the activity of Hypoxia Inducible Factor (HIF)-1α, which plays a significant role in tumour promotion (Lau et al. 2006; Yeo et al. 2004; Zhao et al. 2007). However, this suppressive effect of YC-1 on HIF-1α expression is again proposed to occur independently of the sGC-cGMP pathway (Chun et al. 2001; Yeo et al. 2006).

In summary, YC-1 was found to possess many potentially beneficial therapeutic properties, but further optimization was required to realize its full therapeutic potential. It was used as a lead structure and starting point for further chemical and pharmacological optimization (improving in vitro and in vivo potency and pharmacokinetics, and avoiding cGMP-independent effects and PDE-5 inhibition) to generate a compound, suitable for clinical development. In addition to the next-generation compounds described in the subsequent sections of this chapter, a number of analogues of YC-1 were identified as inhibitors of platelet aggregation by Lee and colleagues. However, PDE-5 inhibitory activity remained a limitation in these compounds (Lee et al. 2001). More recently, a phosphonate analogue of YC-1 with improved aqueous solubility was synthesized. This analogue was shown to stimulate sGC at concentrations typically used in the stimulation of sGC by the original YC-1 compound. With improved solubility properties, the phosphonate analogue was also tested at higher concentrations showing a somewhat unexpected inhibitory effect (Martin et al. 2007).

2.1.2 CFM-1571

CFM-1571 was developed using YC-1 as a lead structure (Selwood et al. 2001).

CFM-1571 is a weak but specific activator of sGC ($EC_{50} = 5.5\,\mu M$) that has been shown to inhibit platelet aggregation ($EC_{50} = 2.8\,\mu M$). It synergizes with NO, with no PDE inhibitory activity and marginal inhibition of NOS. However, it exhibits relatively low oral bioavailability (12%).

2.1.3 BAY 41-2272 and BAY 41-8543

A chemical optimization programme of 2,000 newly synthesized compounds for the development of next-generation sGC stimulators (based on YC-1 as a lead structure) yielded two pyrazolopyridine derivatives, BAY 41-2272 and BAY 41-8543 (Stasch et al. 2002a, 2001; Straub et al. 2001). The mode of action of these two compounds is similar to that of YC-1, but they demonstrate greatly increased potency and specificity for sGC. Their vasodilatory potency is approximately 2–3 orders of magnitude higher than that of YC-1 (Stasch et al. 2002a, 2001; Straub et al. 2001). Metabolites of BAY 41-2272 and BAY 41-8543 retain the ability to activate sGC, and may therefore contribute to the in vivo activity of the two drugs (Straub et al. 2002).

In the absence of NO, BAY 41-2272 stimulates sGC activity approximately 20-fold from baseline (Stasch et al. 2001); BAY 41-8543 exhibits even greater potency, stimulating sGC activity up to 92-fold (Stasch et al. 2002a). Indeed, BAY 41-8543 has demonstrated about 3-fold greater potency than BAY 41-2272 in various in vitro and in vivo assays (Stasch et al. 2002a, 2001; Straub et al. 2001). Both compounds strongly synergize with NO, stabilizing the nitrosyl-haem complex to stimulate sGC activity up to 200-fold (Schmidt et al. 2003).

Photoaffinity labelling studies and deletion analysis suggest interaction of BAY 41-2272 with the α subunit of sGC, similar to YC-1 (Becker et al. 2001; Hering et al. 2006; Koglin et al. 2002; Stasch et al. 2001). However, as discussed for YC-1, co-crystallization studies are required to provide conclusive evidence regarding the regions of sGC involved in direct binding to these next-generation compounds.

In contrast to YC-1 (Galle et al. 1999), BAY 41-2272 was found to be devoid of any significant PDE-5 inhibitory activity at concentrations of up to $10\,\mu M$ (Stasch et al. 2001). BAY 41-8543 and BAY 63-2521 also do not cause inhibition of PDE-5 (Schermuly et al. 2008; Stasch et al. 2002a). Although Mullershausen and colleagues proposed that inhibition of PDE-5 could occur at lower concentrations of BAY 41-2272 than those reported by Stasch and colleagues ($IC_{50} = 3\,\mu M$) (Mullershausen et al. 2004), this remains 1–2 orders of magnitude higher than the concentration required to stimulate sGC ($EC_{50} = 0.09$–$0.17\,\mu M$) produce anti-platelet effects ($IC_{50} = 0.01$–$0.3\,\mu M$) (Hobbs and Moncada 2003, Koglin et al. 2002; Stasch et al. 2001), and cause relaxation in a number of isolated tissues including aorta, saphenous vein, vaginal wall, and corpus cavernosum ($EC_{50} = 0.06$–$0.49\,\mu M$) (Cellek 2003; Hobbs 2002; Kalsi et al. 2003, 2004; Stasch et al. 2001). Indeed, studies using recombinant sGC revealed that

concentrations of BAY 41-2272 as low as 0.01–0.1 μM stimulate sGC to a level that would be expected to cause biologically important increases in cGMP (Stasch et al. 2001). Moreover, BAY 41-2272 and BAY 41-8543 do not inhibit other cGMP-specific/metabolizing PDEs, such as PDE-1, -2, and -9 (Bischoff and Stasch 2004; Stasch et al. 2001, 2002a).

In vivo studies provide further evidence against a role for PDE-5 inhibition in the action of BAY 41-2272. For instance, infusion of BAY 41-2272 in lambs with acute pulmonary hypertension has been shown to cause strong pulmonary vasodilatation, but even the highest plasma concentration achieved in this study (0.06 μM) appeared to be too low to inhibit PDE-5 (Evgenov et al. 2004). Furthermore, the pulmonary vasodilator effects of BAY 41-2272 are not suppressed by the NOS inhibitor L-NAME (Evgenov et al. 2004), whereas pre-treatment with L-NAME completely blocks the pulmonary vasodilatation caused by the PDE-5 inhibitor, sildenafil (Weimann et al. 2000). As such, the vast majority of published literature on BAY 41-2272 argues against any inhibitory effect on PDE-5; for further discussion on this subject, see Bischoff and Stasch (2004). Moreover, sustained sGC stimulation by BAY 41-8543 offset NOS inhibition to restore acute cardiac modulation by PDE-5 inhibitor sildenafil (Nagayama et al. 2008).

Pre-clinical studies have confirmed that BAY 41-2272 and BAY 41-8543 exert potent vasodilatory, anti-platelet, and anti-proliferative activity ex vivo. Vasodilatory effects of BAY 41-2272 and BAY 41-8543 have been observed in isolated systemic, aortic, coronary, and pulmonary arteries and veins, and the compounds potently reduce coronary perfusion pressure in the rat heart Langendorff preparation without any effect on left ventricular pressure and heart rate (Hobbs 2002; Stasch et al. 2002a, 2001). BAY 41-2272 also relaxes rat anococcygeus muscle, rabbit, penile urethra, vaginal wall and corpus cavernosum, and mouse and human penile corpus cavernosum (Baracat et al. 2003; Cellek 2003; Kalsi et al. 2003; Teixeira et al. 2006a, 2007; Toque et al. 2008), and BAY 41-8543 stimulates equivalent vasodilatation in normal and nitrate-tolerant systems (Stasch et al. 2002a), suggesting it may not suffer from the problem of tachyphylaxis. BAY 41-2272 also elicits anti-proliferative actions in smooth muscle cells (Wharton et al. 2005).

BAY 41-2272 prevents aggregation of washed platelets (Hobbs and Moncada 2003; Stasch et al. 2001) and inhibits aggregation in platelet-rich plasma, albeit with distinctly reduced potency compared with washed platelets (Hobbs and Moncada 2003). Moreover, BAY 41-2272 and BAY 41-8543 both inhibit collagen-stimulated aggregation of washed human platelets at concentrations in the nanomolar range (Hobbs and Moncada 2003; Stasch et al. 2002a, 2001). An inhibition of expression and procoagulant activity of tissue factor (TF) by BAY 41-2272 in monocytes and endothelial cells was observed (Evgenov et al. 2008). BAY 41-2272 also induces endothelial cell mobilization, demonstrating a role for sGC in angiogenesis (Pyriochou et al. 2006).

BAY 41-2272 activates the $\alpha 2\beta 1$ heterodimeric sGC in addition to the $\alpha 1\beta 1$ enzyme dimer (Koglin et al. 2002). Interestingly, the $\alpha 2$ subunit is most highly expressed in the brain (Budworth et al. 1999; Mergia et al. 2003) and, akin to YC-1, BAY 41-2272 sensitizes, otherwise NO-unresponsive cells to NO (van Staveren

et al. 2005); the effect of BAY 41-2272 on learning and memory is currently under investigation (Koenig et al. 2004; Luithle et al. 2004; Stahl et al. 2004).

BAY 41-2272 is also efficacious in the treatment of animal models of cardiovascular disease and renal failure, and has potential for the treatment of erectile dysfunction. In rats, L-NAME-induced hypertension is abolished by co-treatment with BAY 41-2272, which also prevents the associated cardiac hypertrophy and fibrosis (Stasch et al. 2001; Zanfolin et al. 2006). BAY 41-2272 also reduces cardiac hypertrophy and prevents vascular remodelling in rats with hypoxia-induced PH compared with untreated controls (Deruelle et al. 2006), and it possesses antiproliferative, anti-fibrotic, and anti-proteinuric effects in rat models of glomerulonephritis (Hohenstein et al. 2005; Peters et al. 2004; Wang et al. 2005b). Indeed, anti-remodelling effects of BAY 41-2272 have been observed at doses that do not alter blood pressure (Masuyama et al. 2006).

In a canine model of congestive heart failure, BAY 41-2272 unloads the heart and increases cardiac output while maintaining glomerular filtration without activating the renin–angiotensin–aldosterone system (Boerrigter et al. 2003). BAY 41-2272 is also efficacious in a canine model of PH induced by heparin-protamine interaction (Freitas et al. 2007). BAY 41-2272 causes potent and sustained pulmonary vasodilatation in healthy foetal lambs (Deruelle et al. 2005b). Furthermore, it causes potent pulmonary vasodilatation and augments the response to inhaled NO in lambs with acute PH caused by administration of the thromboxane analogue U-46619 (Evgenov et al. 2004), and in a sheep model of persistent pulmonary hypertension of the newborn (Deruelle et al. 2005a).

In a more recent study, BAY 41-2272 and BAY 41-8543 were targeted to the pulmonary vasculature directly by inhaled administration of dry-powder, lipid/protein/sugar-based microparticles encapsulating the drugs. This approach caused pulmonary vasodilation in a sheep model of acute PH without affecting mean arterial pressure. Inhalation of BAY 41-8543 increased systemic arterial oxygenation as well as augmenting the magnitude and duration of the pulmonary vasodilatory response to inhaled NO (Evgenov et al. 2007).

BAY 41-2272 may also protect rats against thrombosis and/or stroke; its antiaggregatory properties significantly increase tail bleeding time in rats (Stasch et al. 2001). Stimulation of sGC also has anti-inflammatory actions. BAY 41-2272 inhibits expression of the adhesion molecule P-selectin and reduces leukocyte recruitment in endothelial NOS knockout mice, and mice treated with interleukin-1β (Ahluwalia et al. 2004). Finally, treatment of healthy rabbits with BAY 41-2272 induces penile erection in an NO-dependent manner (Bischoff et al. 2003), suggesting a potential role in the treatment of erectile dysfunction.

BAY 41-8543 was the first sGC stimulator investigated in healthy volunteers and, as expected from the animal studies, induced the desired haemodynamic effects (e.g., lowering of blood pressure). However, due to multiple pharmacokinetic issues a follow-up compound was needed. This led to the discovery of BAY 63-2521, a compound chemically related to the former sGC stimulators (Fig. 1)

2.1.4 BAY 63-2521

The optimized sGC stimulator, BAY 63-2521 was identified following the pharmacological and pharmacokinetic profiling of approximately 1,000 additional compounds in pre-clinical tests (Evgenov et al. 2006). BAY 63-2521 concentration-dependently stimulates sGC activity up to 73-fold in vitro from 0.01 to 100 μM and synergizes with the NO donor drug DEA/NO (0.1 μM) to increase sGC activity up to 112-fold (Schermuly et al. 2008). BAY 63-2521 exhibits similar properties in endothelial cells, acting in synergy with NO to increase intracellular cGMP levels in a concentration-dependent manner.

BAY 63-2521 possesses vasodilatory properties similar to BAY 41-8543 in vitro and in vivo. At concentrations of 0.01–1 μM, the compound reduces coronary perfusion pressure in the rat heart Langendorff preparation without affecting left ventricular pressure and heart rate. It also inhibits contraction of rabbit aortic rings, rabbit saphenous artery rings, porcine coronary artery rings, canine femoral vein rings, and strips of rabbit corpus cavernosum. Furthermore, BAY 63-2521 promotes vasorelaxation in arteries isolated from nitrate-tolerant rabbits, and decreases acute pulmonary vasoconstriction in isolated mouse lungs at a concentration of 0.01 μM (Schermuly et al. 2008). A recent study has revealed that BAY 63-2521 is also effective in reducing PH in mouse and rat models (hypoxia and monocrotaline induced PH, respectively) compared with untreated controls, and decreases the associated right heart hypertrophy and vascular remodelling (Schermuly et al. 2008).

Atherosclerosis has been associated with reduced cGMP signalling particularly in the neointimal layer, suggesting a connection between cGMP signalling and neointimal proliferation and vascular dysfunction (Melichar et al. 2004). Long-term treatment with BAY 63-2521 over 14 weeks (300 ppm in solid feed) decreases atherosclerosis in ApoE knockout mice, reducing cross-sectional plaque area at the aortic root to 53% of that observed in untreated controls. BAY 63-2521 also inhibits human coronary artery smooth muscle cell migration, which is an early feature of atherosclerosis.

At concentrations up to 10 μM, BAY 63-2521 neither inhibits the cGMP-specific PDEs, PDE-5 and PDE-9, nor inhibit the cGMP metabolizing PDEs, PDE-1 and PDE-2 (Schermuly et al. 2008). Therefore, the vasodilator activity of BAY 63-2521 is not mediated by inhibition of these PDEs. BAY 63-2521 has now undergone phase 1 and proof-of-concept clinical trials, and phase 2 trials are currently underway (see Sect. 4).

2.2 The Aryl-Acrylamide Family

Another class of sGC stimulator (acrylamide analogues) was discovered independently of YC-1. These compounds were found to be structurally dissimilar to YC-1, but they utilize a similar mode of action, acting independently of and in synergy with NO and requiring the presence of a reduced haem moiety (Miller et al. 2003;

Nakane 2003; Nakane et al. 2006; Zhang et al. 2003). Aryl-acrylamide compounds cause relaxation of cavernosum tissue strips in vitro and induction of penile erection in rats (Miller et al. 2003; Nakane et al. 2006).

3 Future Directions and Therapeutic Applications

Experimental animal models have demonstrated potential therapeutic roles for sGC stimulators in a range of cardiovascular and non-cardiovascular disorders.

3.1 Hypertension

Hypertension is a critical public health issue in the developed world; nearly 1:3 adults have hypertension in the USA, and its prevalence is increasing with time (Fields et al. 2004; Rosamond et al. 2007). The total healthcare costs of hypertension in 2007 was estimated to be $66.4 billion (Rosamond et al. 2007). Hypertension can lead to stroke, heart disease, and renal failure (Messerli et al. 2007) and remains one of the leading causes of mortality in the USA (Kung et al. 2007).

Hypertension is associated with changes in the NO-sGC-cGMP signalling pathway. Maximal cGMP production by sGC from lungs of spontaneously hypertensive (SHR) rats is reduced approximately threefold compared with control Wistar-Kyoto (WKY) animals (Kojda et al. 1998), and the EC_{50} for sodium nitroprusside is increased threefold in SHR compared with wild-type (WKY) rats (Kloss et al. 2000). Expression of sGC is also reduced in SHR compared with wild-type rats (Kloss et al. 2000; Morawietz et al. 2001; Ruetten et al. 1999). Endothelial cells have been shown to mount a compensatory response in hypertensive rats by enhancing NO production (Kagota et al. 2006). However, this may contribute to sGC desensitization. Chronic deficiency of NO in eNOS knockout mice and acute cessation of endothelial NO synthesis in wild-type mice increases the sensitivity of sGC to NO and enhances vascular smooth muscle cell relaxation in response to nitrovasodilator agents, whereas high ambient NO concentrations induce a desensitization of the enzyme (Brandes et al. 2000; Hussain et al. 1999).

The use of classical NO donor drugs to treat hypertension is hampered not only by nitrate tolerance, but also by the oxidative stress associated with hypertension. Cardiovascular diseases, including hypertension, are often associated with elevated levels of angiotensin II, which stimulates NAD(P)H oxidase to increase production of superoxide (Harrison et al. 2003). This reacts with NO to form peroxynitrite, thus reducing the level of NO available to stimulate sGC (Chirkov and Horowitz 2007; Franco and Oparil 2006; Munzel et al. 2005). However, stimulation of sGC in an NO-independent manner is not subject to these limitations, and therefore has a significant advantage over existing NO donor drugs. Thus, administration of multiple doses of sGC stimulators does not cause tachyphylaxis. NO-independent stimulators

of sGC have been shown to lower blood pressure and increase survival in hypertensive rats (Stasch et al. 2001, 2002b), and act in synergy with sodium nitroprusside (Rothermund et al. 2000). BAY 41-2272 also reverses the cardiovascular complications associated with hypertension (cardiac hypertrophy and fibrosis) in rat models of the disease (Masuyama et al. 2006; Stasch et al. 2001; Zanfolin et al. 2006).

3.2 Pulmonary Hypertension

Pulmonary hypertension (PH) is a devastating disease in which increased pulmonary vascular resistance causes right heart hypertrophy, eventually leading to right heart failure and death (Gaine and Rubin 1998); without treatment, median life expectancy following diagnosis is 2.8 years (D'Alonzo et al. 1991). PH exists in many different forms, which are categorized into groups according to their aetiology. Categories of PH include pulmonary arterial hypertension (PAH), PH with left heart disease, PH associated with lung diseases and/or hypoxaemia, and PH due to chronic thrombotic and/or embolic disease (Simonneau et al. 2004). PAH is rare in the general population (30–50 cases per million) (Peacock 2003), but the prevalence of PAH increases in association with certain common conditions such as HIV infection (0.5%) (Speich et al. 1991), scleroderma (10–15%) (Denton and Nihtyanova 2007), and sickle cell disease (30%) (Aliyu et al. 2008). Other forms of PH are generally more common than PAH, and the association of PH with chronic obstructive pulmonary disease (COPD) is of particular concern, due to the high prevalence of COPD. Approximately 1% of the general population is estimated to have COPD, and this figure rises to more than 10% among those aged 40 years or more (Chapman et al. 2006). COPD is one of the leading causes of death worldwide (Pauwels and Rabe 2004), and its prevalence and mortality are projected to increase with time (Murray and Lopez 1997), largely as a consequence of the continued use of tobacco and the ageing of the global population (Chapman et al. 2006). The development of PH is a poor prognostic sign in patients with COPD, and it occurs at a relatively high frequency. In a study of 131 patients with mild to moderate hypoxaemic COPD, 25% went on to develop PH over a period of six years (Kessler et al. 2001).

In PAH, the bioactivity of NO and other vasodilators such as prostacyclin is reduced (Giaid and Saleh 1995; Tuder et al. 1999), whereas production of endogenous vasoconstrictors such as endothelin is increased (Luscher and Barton 2000), resulting in excessive pulmonary vasoconstriction. Treatments that elevate NO levels (inhaled NO and NO donor drugs) are unsuitable as long-term therapies for PAH, due to their short-lived effects and the development of tolerance. Precise regulation of NO levels is required in the pulmonary vasculature, to direct blood flow preferentially to well-ventilated regions of the lung (ventilation/perfusion matching), thus ensuring optimal uptake of oxygen into the blood (Ghofrani et al. 2006). Therefore, therapies that act in synergy with endogenous NO to maintain ventilation/perfusion matching are highly desirable.

The PDE-5 inhibitor sildenafil augments the effects of endogenous NO, increasing cGMP levels by preventing its degradation. Though this approach overcomes the shortcomings associated with NO-based therapies, a significant proportion of patients with PAH do not respond to treatment with sildenafil (Chockalingam et al. 2005), indicating that endogenous NO (and therefore the rate of cGMP synthesis) in these patients is decreased to such an extent that sildenafil can no longer raise cGMP levels to a sufficient degree. Direct stimulation of sGC represents a promising alternative therapeutic strategy for such patients.

Stimulation of sGC causes pulmonary vasodilation and enhances the response to inhaled NO in ovine and canine models of PAH (Deruelle et al. 2005a; Evgenov et al. 2004; Freitas et al. 2007), and the vasodilatory response to BAY 41-2272 is prolonged in comparison with sildenafil (Deruelle et al. 2005a). BAY 41-2272 also promotes vasodilation in a canine model of acute pulmonary embolism (Cau et al. 2008), a major cause of acute PH and subsequent acute right heart failure, which is thought to be driven at least in part by pulmonary vasoconstriction (Layish and Tapson 1997; Smulders 2000). Stimulation of sGC may reverse the structural changes associated with PAH; BAY 41-2272 has anti-proliferative effects in human pulmonary arterial smooth muscle cells in vitro (Wharton et al. 2005). Treatment with BAY 41-2272 reduces vascular remodelling and right heart hypertrophy in rodent models of PAH (Deruelle et al. 2006; Dumitrascu et al. 2006). A recent study in a lamb model of PAH demonstrated the feasibility of targeting sGC stimulators directly to well-ventilated regions of the lung via inhalation (Evgenov et al. 2007); this approach stimulated pulmonary vasodilatation and increased oxygenation without significantly affecting mean arterial pressure.

3.3 Heart Failure

Heart failure is defined as an impaired ability of the ventricle to fill with or eject blood, and is characterized by symptoms including dyspnoea and fatigue. It is a serious and growing health concern; in the USA alone, approximately 5 million people have heart failure, with more than 0.5 million new cases being diagnosed each year. Heart failure caused more than 50,000 deaths in 2001, and the annual number of deaths due to heart failure is increasing despite advances in treatment. The total annual cost associated with heart failure in the USA has been estimated to be $30 billion (Hunt et al. 2005).

The development of heart failure involves abnormal signalling in a number of pathways, including inadequate release of atrial and B-type natriuretic peptides (both particulate GC ligands) as well as reduced NO-mediated signalling (Boerrigter and Burnett 2004; Hare and Stamler 2005). Activation of sGC in the NO signalling pathway is reduced by several mechanisms in heart failure, including decreased production and increased degradation of NO, and interaction of NO with superoxide to form peroxynitrite (Hare and Stamler 2005). In addition to reducing NO signalling, the generation of peroxynitrite also directly impairs cardiovascular function, for

example, by activating matrix metalloproteases and the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Pacher et al. 2005; Ungvari et al. 2005). NO donor drugs restore NO levels in heart failure and have been used to treat the disease for over a century, but their long-term use is limited by the development of tolerance (Munzel et al. 2005). Furthermore, bioactivation of the NO donor drug, glyceryl trinitrate, has been reported to occur at a higher rate in venous blood vessels than in arterial blood vessels (Mulsch et al. 1995), which could lead to a detrimental decrease in right atrial pressure. Stimulation of the NO signalling pathway independent of NO therefore represents an attractive alternative for the treatment of heart failure. In support of this, stimulation of sGC with BAY 41-2272 unloads the heart, increases cardiac output and, in contrast to glyceryl trinitrate, maintains right atrial pressure in a canine model of heart failure (Boerrigter et al. 2003).

3.4 Atherosclerosis, Restenosis, and Thrombosis

Atherosclerosis is an inflammatory disease characterized by arterial lesions (atheromata), which consist of cells (particularly inflammatory and immune cells), lipids, connective tissue materials such as collagen and elastin, and debris. Atheromata are thought to be triggered by haemodynamic strain and lipid accumulation in the arterial intima, which initiate an inflammatory process involving adhesion of platelets and recruitment of monocytes. The latter differentiate into macrophages and release factors that cause further inflammation and tissue damage, promoting proliferation and migration of smooth muscle cells. Thrombosis and ischemia occur when an atheroma ruptures, exposing pro-thrombotic material to the blood (Hansson 2005). Development of atherosclerosis begins early in life (Strong et al. 1999) and is a major risk factor for cardiovascular disease, which continues to be a leading cause of death in the USA (Rosamond et al. 2007).

NO prevents platelet aggregation and endothelial cell proliferation in addition to promoting vasodilatation. Decreased bioavailability of endothelial NO plays a critical role in the development of atherosclerosis. Endothelial NO levels are reduced in atherosclerosis as a result of scavenging by superoxide and reduced expression and activity of eNOS (Yang and Ming 2006); in addition, expression and activity of sGC are reduced specifically in atherosclerotic lesions (Melichar et al. 2004). The potential of sGC as a target for pharmacological therapy in atherosclerosis has therefore been explored.

NO-independent sGC stimulation by YC-1 attenuates balloon injury-induced neointima formation in rat arteries (Tulis et al. 2000; Wu et al. 2004), and prevents platelet aggregation and smooth muscle cell proliferation (Tulis et al. 2002). BAY 41-8543 and BAY 41-2272 also exert anti-aggregatory and anti-proliferative effects (Hobbs and Moncada 2003; Stasch et al. 2002a, b, 2001), and BAY 41-2272 inhibits leukocyte recruitment in mice (Ahluwalia et al. 2004). Finally, BAY 63-2521 decreases atherosclerosis in ApoE knockout mice, and inhibits human coronary artery smooth muscle cell migration.

3.5 Erectile Dysfunction

Erectile dysfunction, defined as the "inability to achieve or maintain an erection adequate for sexual satisfaction" (National Institutes of Health 1993), has a prevalence ranging from 2% in men under 40 years of age to 86% in men aged 80 years or more (Prins et al. 2002). Penile erection is a complex process involving central nervous regulation (spinal and supraspinal) and peripheral regulation by sympathetic, parasympathetic, somatic, and sensory nerves. One important population of regulatory peripheral nerves contains NOS, and the NO-sGC-cGMP pathway plays an essential role in the process of penile erection by translating non-adrenergic, non-cholinergic (NANC) neurotransmissions into smooth muscle relaxation in the corpus cavernosum (Andersson 2001; Ignarro et al. 1990; Rajfer et al. 1992). NO release from nitrergic nerves is thought to be impaired in patients with diabetes, giving rise to diabetes-induced erectile dysfunction; in support of this, rats with diabetes induced by streptozotocin have reduced nitrergic relaxation responses (Kalsi et al. 2004).

Pharmacological agents that increase intracellular cGMP levels have therefore attracted much interest, and PDE inhibitors such as sildenafil are now widely used to treat erectile dysfunction. However, up to 30% of patients with erectile dysfunction do not respond to treatment with sildenafil (Ghofrani et al. 2006), indicating that NO levels in these patients are decreased to such an extent that sildenafil can no longer raise cGMP levels to a sufficient extent, similar to the situation in PAH. NO-independent stimulation of sGC may represent an attractive alternative for these patients.

NO-independent sGC stimulators promote smooth muscle relaxation in the corpus cavernosum of rats, rabbits, and humans and cause penile erection in vivo in rats and rabbits (Baracat et al. 2003; Bischoff et al. 2003; Kalsi et al. 2003; Miller et al. 2003; Mizusawa et al. 2002; Nakane et al. 2002). Furthermore, BAY 41-2272, but not sildenafil, enhances residual nitrergic relaxation responses in the anococcygeus muscle of streptozotocin diabetic rats. This suggests that NO-independent sGC stimulation may have greater efficacy than PDE-5 inhibition in the treatment of patients with diabetes-induced erectile dysfunction (Kalsi et al. 2004). NO-independent sGC stimulators have also been shown to act in synergy with the NO donor, sodium nitroprusside, to produce penile erection in rabbits, implying that these compounds will enhance the response to endogenous NO released during sexual stimulation and thus facilitate a natural penile erection (Bischoff et al. 2003; Nakane et al. 2002). Recent data suggest that BAY 41-2272 may also reduce superoxide formation and NADPH oxidase expression in the corpus cavernosum (Teixeira et al. 2007), thereby increasing the bioavailability of NO.

3.6 Renal Fibrosis

Renal fibrosis is a devastating condition, which occurs as a result of a variety of chronic kidney diseases. It is characterized by widespread tissue scarring and the destruction of functional tissues, eventually leading to end-stage renal failure that requires dialysis or kidney transplantation. Extracellular matrix accumulation secondary to increased expression of transforming growth factor β (TGFβ) plays a key role in the pathogenesis of renal failure (Gaedeke et al. 2006). NO donor drugs have been shown to prevent matrix accumulation (Peters et al. 2003), and signalling via sGC has been identified as an important anti-fibrotic pathway (Peters et al. 2004). Furthermore, sGC expression and cGMP levels are increased in a rat model of mesangial proliferative glomerulonephritis, possibly as part of an endogenous protective mechanism (Hohenstein et al. 2005; Peters et al. 2004). The sGC-cGMP signalling pathway has therefore attracted interest as a potential therapeutic target in renal disease.

Compared with a placebo, NO-independent stimulation of sGC with BAY 41-2272 reduces mesangial proliferation, matrix expansion, and proteinuria in a rat model of mesangial proliferative glomerulonephritis (Hohenstein et al. 2005; Peters et al. 2004). BAY 41-2272 also limits disease progression in a rat model of chronic glomerulonephritis and increases renal function relative to controls (Wang et al. 2005b); furthermore, this sGC stimulator has greater efficacy than pentoxifylline, an inhibitor of cGMP degradation (Wang et al. 2006). Importantly, the positive effects of BAY 41-2272 in these studies occur independently of any effect on systemic blood pressure.

3.7 Possible Further Applications

PH can be associated with idiopathic pulmonary fibrosis, in which repeated microscopic injury and failed repair of the alveolar epithelium cause proliferation of interstitial fibroblasts, leading to the development of fibroblast/myofibroblast foci and lung fibrosis (Dempsey et al. 2006; Patel et al. 2007). Lung tissue remodelling and fibrosis are also features of COPD (Postma and Timens 2006). In addition to relieving PH by promoting vasodilatation, sGC stimulation has also been shown to prevent the conversion of human lung fibroblasts into myofibroblasts, and may therefore have dual beneficial effects in PH associated with lung fibrosis (Dunkern et al. 2007). Furthermore, cGMP elevation may also have potential in the treatment of liver fibrosis; for example, the sGC activator BAY 60-2770 reduces collagen accumulation in rodent models of liver cirrhosis (Knorr et al. 2008).

The smooth muscle relaxant properties of sGC stimulators may be advantageous in the treatment of benign prostatic hyperplasia (BPH) and lower urinary tract symptoms (LUTS). For example, the PDE-5 inhibitors sildenafil, tadalafil, and vardenafil induce significant relaxation in rat LUT tissues, inhibit the proliferation of human prostate stromal cells, and reduce irritative LUTS (non-voiding contractions) in pre-

clinical studies (Filippi et al. 2007; Tinel et al. 2006; Toque et al. 2008). Recent clinical studies also revealed a reduction in LUTS in patients with BPH treated with sildenafil and tadalafil over a period of 12 weeks (Sandner et al. 2007).

Stimulators of sGC may also have therapeutic potential in the field of learning and memory, for example in the treatment of dementia and Alzheimer's disease (Feil and Kleppisch 2008; Garthwaite 2008). YC-1 elicits cGMP synthesis in NO-unresponsive GABAergic and glutamatergic cells (van Staveren et al. 2005), and it is therefore possible that YC-1 may enhance long-term potentiation (Bredt 2003; Chien et al. 2003, 2005; 2008). The role of next-generation compounds in learning and memory is currently under investigation.

An anti-inflammatory role has been ascribed to sGC stimulators. For example, stimulation of sGC (either with NO donors or sGC stimulators) has been shown to block inflammatory responses and inhibit leukocyte recruitment (Ahluwalia et al. 2004; Lu et al. 2007; Pan et al. 2005a). Moreover, it has been suggested that inhibition of tissue factor (TF) expression and activity by sGC stimulators might provide therapeutic benefits in inflammation and cardiovascular diseases (Evgenov et al. 2008). The potential of sGC stimulators as anti-cancer agents has also been studied. An analogue of YC-1 that stimulates differentiation and apoptosis in HL-60 cells is now being investigated as a potential therapy for leukaemia (Chou et al. 2007). Moreover, YC-1 induces apoptosis of human renal carcinoma cells in vitro and in vivo (Wu et al. 2008). Recently, YC-1 was found to induce expression of heat shock protein 70 (HSP70) in vascular smooth muscle cells, protecting them against cytotoxicity (Liu et al. 2008); sGC stimulators may thus act as cytoprotective agents in vascular diseases. However, induction of HSP70 again appeared to occur independently of cGMP, and it remains to be established, if more specific next-generation sGC stimulators retain this function.

4 Clinical Trials of the sGC Stimulator BAY 63-2521

Of the sGC stimulators described, BAY 63-2521 alone has made the transition from animal models to patients in clinical trials. Its synergistic action with endogenous NO ensures that vasodilatation is stimulated, preferentially in well-ventilated regions of the lung, thus preventing ventilation/perfusion mismatch. This property renders it an ideal candidate for development as a therapy for patients with PH.

BAY 63-2521 has a favourable safety profile and is well tolerated in healthy volunteers (Frey et al. 2008) and in patients with PH (including PAH, PH associated with interstitial lung disease [ILD] and chronic thromboembolic PH) (Ghofrani et al. 2007). Oral BAY 63-2521 was efficacious in a proof-of-concept study of 15 patients with PH, reducing pulmonary vascular resistance and increasing cardiac output to a significantly greater extent than inhaled NO (Ghofrani et al. 2007) at doses of 1 mg and 2.5 mg. Neither doses produced any deterioration in gas exchange, indicating that ventilation/perfusion matching was maintained. Peak concentrations of BAY 63-2521 in the circulation were reached 0.25–1.5 h following administration of the

drug, and its half-life was between 10 and 12 h. Long-term treatment with BAY 63-2521 continues to alleviate PH symptoms and remains well tolerated, and a full phase 2 study in patients with PAH, PH associated with ILD, and PH associated with COPD is nearing completion.

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NO- and Haem-Independent Soluble Guanylate Cyclase Activators

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Abstract Oxidative stress, a risk factor for several cardiovascular disorders, interferes with the NO/sGC/cGMP signalling pathway through scavenging of NO and formation of the strong intermediate oxidant, peroxynitrite. Under these conditions, endothelial and vascular dysfunction develops, culminating in different cardio-renal and pulmonary-vascular diseases. Substituting NO with organic nitrates that release NO (NO donors) has been an important principle in cardiovascular therapy for more than a century. However, the development of nitrate tolerance limits their continuous clinical application and, under oxidative stress and increased formation of peroxynitrite foils the desired therapeutic effect. To overcome these obstacles of nitrate therapy, direct NO- and haem-independent sGC activators have been developed,

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such as BAY 58-2667 (cinaciguat) and HMR1766 (ataciguat), showing unique biochemical and pharmacological properties. Both compounds are capable of selectively activating the oxidized/haem-free enzyme via binding to the enzyme's haem pocket, causing pronounced vasodilatation. The potential importance of these new drugs resides in the fact that they selectively target a modified state of sGC that is prevalent under disease conditions as shown in several animal models and human disease. Activators of sGC may be beneficial in the treatment of a range of diseases including systemic and pulmonary hypertension (PH), heart failure, atherosclerosis, peripheral arterial occlusive disease (PAOD), thrombosis and renal fibrosis. The sGC activator HMR1766 is currently in clinical development as an oral therapy for patients with PAOD. The sGC activator BAY 58-2667 has demonstrated efficacy in a proof-of-concept study in patients with acute decompensated heart failure (ADHF), reducing pre- and afterload and increasing cardiac output from baseline. A phase IIb clinical study for the indication of ADHF is currently underway.

1 Haem-Free Soluble Guanylate Cyclase as a New Target

Soluble guanylate cyclase (sGC) was therapeutically utilized long before its discovery. NO donor compounds, such as organic nitrates and nitroprusside, promote vasodilatation and inhibit platelet aggregation by stimulating sGC and the formation of cGMP (Murad 2006). Is this target enzyme, sGC, along with NO-cGMP signalling in general, affected under different disease conditions? Is cGMP diminished? Clearly, NO formation and bioavailability are reduced in hypertension, atherosclerosis, heart failure and diabetes. It has been assumed for many years that signalling beyond NO is unaffected. However, recent data suggest that sGC expression or function can be pathologically reduced or even increased (Ruetten et al. 1999; Melichar et al. 2004; Schermuly et al. 2008). In parallel, it became clear that we are only at the beginning of understanding how this enzyme is regulated. There are several alternative activation and modulating mechanisms beyond the binding of NO to the Fe^{2+} haem of sGC's β subunit (Zabel et al. 2002; Nedvetsky et al. 2008; Murthy 2008; Mingone et al. 2008; Meurer et al. 2008). One key observation was the existence of NO-insensitive (haem-oxidized or haem-free) sGC under physiological conditions, and increased levels in certain disease conditions (Stasch et al. 2006). Similar to organic nitrates, which led to the discovery of sGC and endogenous NO, the discovery of this impaired state of sGC was also enabled by a class of drugs with a most unusual pharmacological and biochemical profile, sGC activators (Stasch et al. 2002a).

In vitro, there is a solid body of evidence that sGC can exist in an oxidized/haem-free state and sGC activators such as BAY 58-2667 or HMR1766 selectively bind to this form with extremely high affinity, but not to (Fe^{2+}) haem-containing sGC. In vivo, evidence for the existence of oxidized/haem-free sGC is circumstantial. However, the in vivo and in vitro profiles of sGC activators are indistinguishable; thus the physiological existence of oxidized/haem-free sGC can no longer be rejected. A useful set of pharmacological tools to modulate these NO-insensitive states of

sGC are now available: full agonists, such as BAY 58-2667, partial agonists, such as protoporphyrine IX (PPIX; Ignarro et al. 1982, 1984) and a full antagonist, Zn-PPIX (Stasch et al. 2006). Zn-PPIX will not affect NO-induced Fe^{2+}-sGC activation. Why there is oxidized/haem-free sGC under physiological conditions and why it seems to be even further increased under disease conditions is unclear. The fact that the haem oxidant, ODQ, also leads to a BAY 58-2667 sensitive form of sGC in vitro and in vivo suggests that a similar oxidative mechanism in diseased blood vessels represents the underlying pathomechanism. However, whether oxidative stress always correlates with the occurrence of oxidized/haem-free sGC remains to be shown; alternative mechanisms that may yield haem-free sGC include deficient haem biosynthesis or incorporation (Mingone et al. 2008). Haem-free sGC seems to be a physiological phenomenon suggesting that there may also be a physiological role and activator compound and thus an entire NO-independent signalling function for haem-free sGC. In fact PPIX may be considered the first such endogenous agonist of haem-free sGC (Mingone et al. 2006; Perkins 2006).

sGC activators are also the first pure sGC activating agents that do not rely on NO. Haem-dependent sGC stimulators may still act in vivo by augmenting endogenous NO; sGC activators are only additive to NO but neither augment NOs' effects nor vice versa. They also do not seem to cause tolerance as NO donors do; in fact under some conditions, they even increase sGC expression. Thus with the use of sGC activators, the long term outcome of chronic cGMP elevation can now be studied via this haem-free sGC pathway and in the absence of NO. Moreover, chronic therapy, which was hitherto obsolete due to NO donor tolerance or limited to parenterally applied natriuretic peptides, seems now a realistic option.

2 Role of Haem in sGC Function

From an early stage on it was clear that the major point of attack for the gaseous messenger nitric oxide (NO) is the reduced haem moiety of sGC, because removal of the prosthetic group abolished any NO-induced enzyme activation (Ignarro et al. 1986; Foerster et al. 1996).

The haem group of sGC is bound to the β-subunit via its axial ligand His^{105} (Wedel et al. 1994; Zhao et al. 1998) and the unique sGC-haem-binding motif Tyr^{135}–Ser^{137}–Arg^{139} (Y–S–R), which is the counterpart of the haem propionic acid groups (Pellicena et al. 2004; Schmidt et al. 2004; Schmidt et al. 2005; Rothkegel et al. 2006, 2007). Binding of NO to the haem results in the formation of a penta-coordinated nitrosyl-haem-complex, indicating that cleavage of the haem-histidine^{105} bond is the molecular switch that leads to the activation of the enzyme. This early binary model of sGC activation was also supported by reconstitution studies with the iron-free precursor of haem, PPIX, whose inability to form the bond to the His^{105} resulted in the activation of the enzyme (Ignarro et al. 1982). The hypothesis that the cleavage of the histidyl-haem bond is the major molecular mechanism to activate sGC was also in agreement with the observed weak activation of sGC by CO, which forms a stable six-coordinated state (Friebe et al. 1996).

However, as the resolution of the applied techniques increased within the recent years it has become evident that the binary model of sGC activation does not fit the observed activation characteristics of the enzyme. Makino and co-workers showed that addition of NO results in the formation of a hexa-coordinated intermediate state, which is subsequently converted into the penta-coordinated active state (Makino et al. 1999). This model was further extended by the observation that this transformation depends on the concentration of free NO suggesting that a second binding site for NO might exist (Zhao et al. 1999). Russwurm and co-workers confirmed this result and reported that sGC, although wavelength scans detected the formation of the nitrosyl-haem-complex, can exist in a virtually inactive form (Russwurm and Koesling 2004). This low-activity sGC state, which is formed in the presence of stoichiometric concentrations of NO, can be transformed by additional NO into the fully activated enzyme (Russwurm and Koesling 2004; Cary et al. 2005). Recent studies using *N*-butyl isocyanide (BIC) as a weak haem-binding sGC activator showed that additional free NO is able to increase the weak BIC-induced sGC activation although this compound prevents NO from interacting with the haem group (Derbyshire and Marletta 2007). One possible alternative for an interaction of NO with sGC beside the prosthetic haem might be the nitrosation of cysteines as shown recently by Sayed and co-workers (Sayed et al. 2007). Whether the identified NO-bound low-active state of sGC represents a novel cellular mechanism of sGC regulation or an in vitro finding without impact under physiological conditions remains to be clarified. Recent work published by Roy and Garthwaite was not able to verify the existence of the proposed low-active state in cellular assays (Roy and Garthwaite 2006). More than two decades after the formulation of the initial hypothesis of NO-mediated sGC activation, the exact mechanism remains an open question and a challenging topic for further research.

3 NO- and Haem-Independent sGC Activators

Our knowledge of sGC as the most important NO receptor has increased tremendously over the last years. About a decade ago, a review was published under the title "sGC: The Forgotten Sibling" (Hobbs 1997). A couple of reasons were given why guanylate cyclase remained poorly defined compared with the well-characterized adenylate cyclase. The recent discovery of novel classes of compounds that activate sGC independently of NO release permits the targeting of a venerable 160-year-old pharmacological target from a completely different angle. NO-independent but haem-dependent sGC stimulators, as well as NO- and haem-independent sGC activators, are valuable tools to elucidate the physiology and pathophysiology of the important NO/sGC signalling pathway in more detail. On the basis of their wide clinical importance, pharmacological in vitro and in vivo results and first clinical studies with these novel compounds, expectations are high. The therapeutic potential of the NO- and haem-independent sGC activators described here was first highlighted in an editorial titled "sGC: An Old Therapeutic Target Re-visited" (Hobbs

2002) and recently at the "International Conference on cGMP Generators, Effectors and Therapeutic Implications" held in Dresden, Germany (Feil and Kemp-Harper 2006; Kemp-Harper and Feil 2008; www.cyclicgmp.net).

3.1 BAY 58-2667 (Cinaciguat) and HMR1766 (Ataciguat)

3.1.1 Discovery and Structure–Activity Relationship

A rapid, homogeneous, cell-based and highly sensitive assay for cGMP that is suitable for fully automated ultra-high-throughput screening (uHTS) and identification of modulators of the NO/cGMP pathway has been established (Wunder et al. 2005). This assay system is based on the cGMP-induced Ca^{2+} influx via the olfactory cyclic nucleotide-gated cation channel CNGA2, acting as the intracellular cGMP sensor. In turn, the intracellular changes in Ca^{2+} levels are translated into alterations of calcium-dependent aequorin bioluminescence. This cell line was used to screen about 2,300,000 compounds in an automated uHTS assay using 1,536-well microtiter plates. In this way, a new class of sGC activators based on the primary uHTS hit, BAY W 1449 (an amino dicarboxylic acid), has been identified. (Stasch et al. 2002a; Wunder et al. 2005). A chemical derivatization program was initiated, and through a series of approximately 800 analogs, BAY 58-2667, one of the most potent representatives of this structural class, was identified to directly activated sGC in an NO-independent manner. The structure–activity relationship is shown in Fig. 1 (Hahn et al. 2007). BAY 58-2667 stimulated aequorin luminescence with a minimal effective concentration (MEC) of 0.3 nM and an EC_{50} value of 10 nM. In the presence of ODQ, the luminescence signal and cGMP production was enhanced and the EC_{50} value was shifted to 1.5 nM (Wunder et al. 2005). BAY 58-2667 relaxes precontracted rabbit saphenous arteries with an IC_{50} value in the subnanomolar range and thus belongs to the most potent relaxing agents (Fig. 1).

Very recently, another sGC activator and close chemical analogue of BAY 58-2667, BAY 60-2770, has been described (Fig. 2). Moreover, the biochemical characterization of two closely related anthranilic acid derivatives, HMR1766 and S3448, has been published, exhibiting comparable characteristics to the dicarboxylic acids BAY 58-2667 and BAY 60-2770, though their chemical structures show no similarities (Schindler et al. 2006; Knorr et al. 2008). Together, these compounds constitute the novel class of NO- and haem-independent sGC activators (Fig. 2).

3.1.2 Mode of Action

BAY 58-2667

Beside the well established NO-mediated as well as NO-independent sGC stimulation by compounds derived from YC-1 (e.g. BAY 41-2272), a further structurally

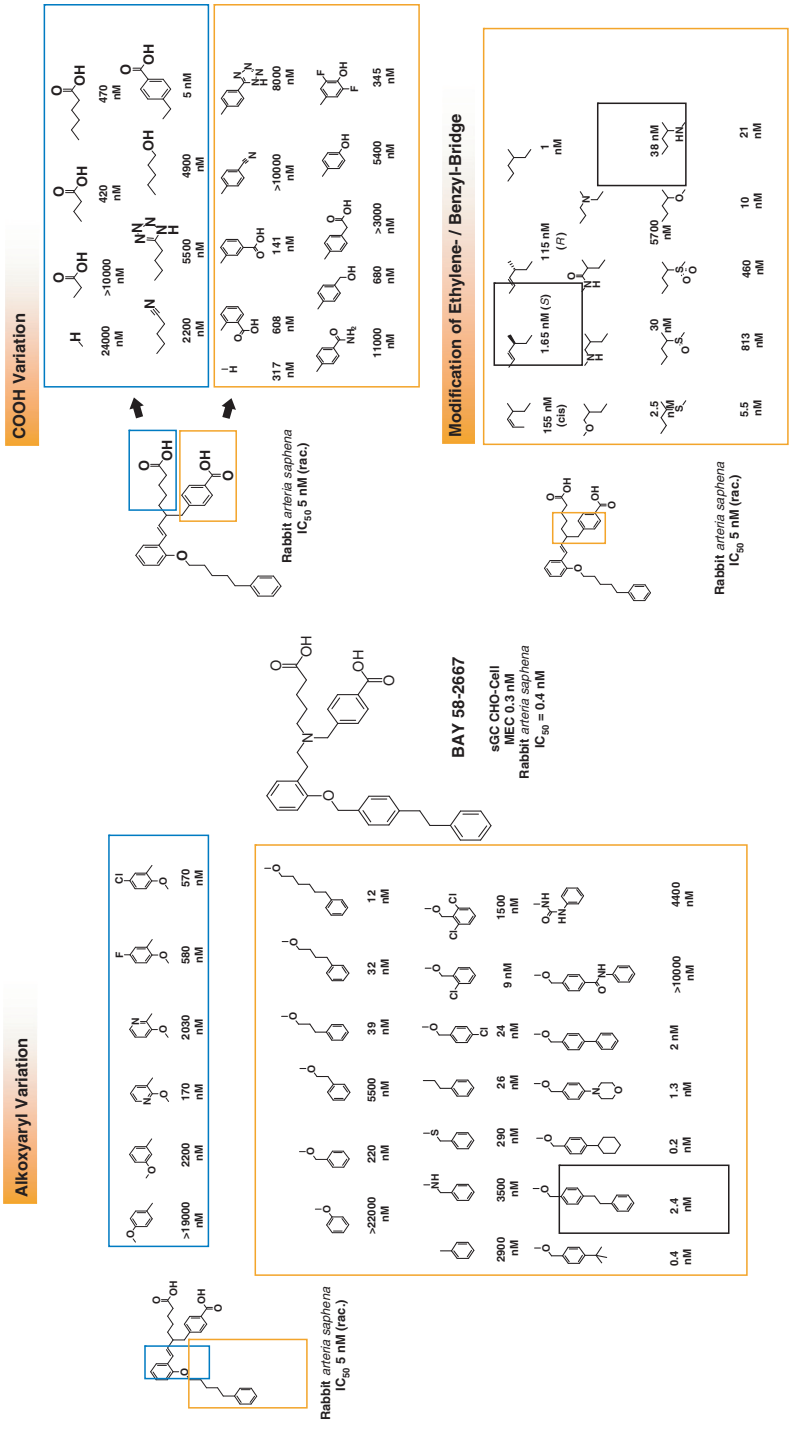

Fig. 1 Structure–activity relationships of dicarboxylic acids

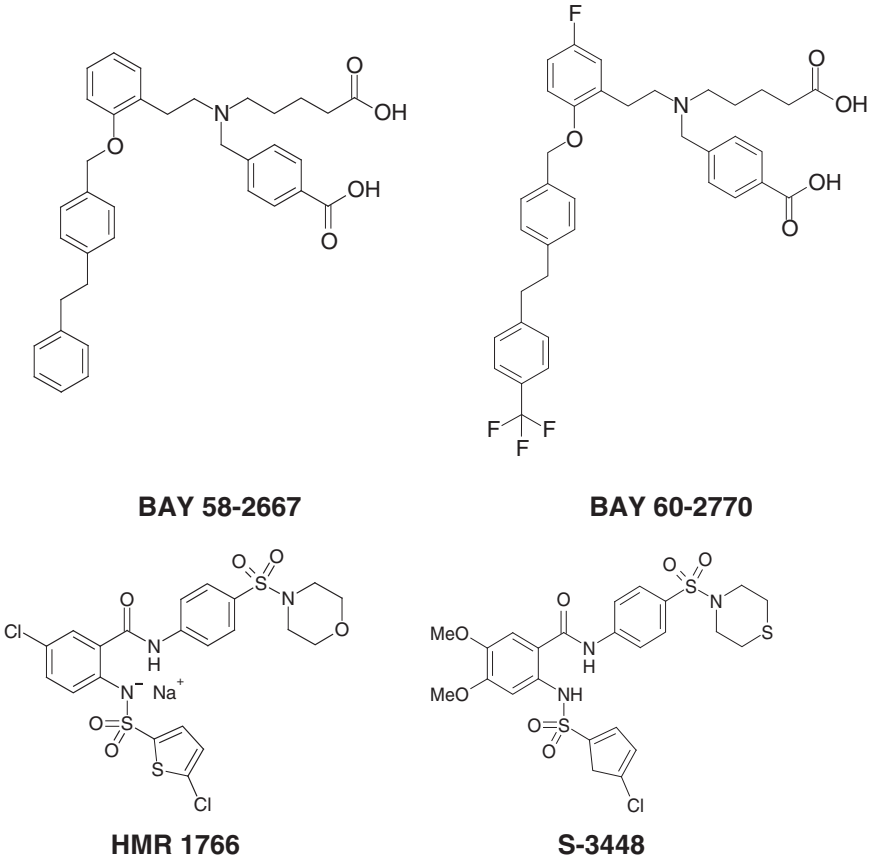

Fig. 2 Chemical structures of sGC activators

unrelated sGC-activating compound was described in 2002 that exhibited unexpected characteristics with respect to enzyme activation (Stasch et al. 2002a). This compound, BAY 58-2667, activated purified sGC preparations to a maximum of about 35-fold and both the binding constant and the EC_{50} value for BAY 58-2667 were shown to be in the low nanomolar range indicating that BAY 58-2667 is the most potent non-NO sGC activator reported so far (Stasch et al. 2002a; Schmidt et al. 2003; Hahn et al. 2007).

In contrast to haem-dependent sGC stimulators such as BAY 41-2272 which strongly synergize with NO, BAY 58-2667 showed only an additive effect when combined with different concentrations of NO-donors (Stasch et al. 2002a; Schmidt et al. 2003). Kinetic analysis revealed that the activation of sGC by this novel structural class was basically reflected by a strong increase in the maximum catalytic rate, whereas only minor changes were observed for the corresponding K_M value for GTP, which decreased from 74 to 56 μM (Schmidt et al. 2003). In particular, the additive effect of BAY 58-2667 and NO-donors entirely originated from addition of

the respective catalytic rates. These first results indicated that, in contrast to the enzyme activation mechanisms of NO and BAY 41-2272, which seem to be somehow interwoven, NO and BAY 58-2667 activate sGC via independent mechanisms.

However, the most surprising finding was that removal of the prosthetic haem by detergents (Foerster et al. 1996) or its oxidation to the NO-insensitive Fe^{3+} state by the sGC inhibitor ODQ (Zhao et al. 2000) strongly potentiated BAY 58-2667-induced enzyme activation (Stasch et al. 2002a). ODQ resulted in a concentration-dependent increase of BAY 58-2667-induced sGC activation (EC_{50} ODQ: 170 nM) that was accompanied by an increased binding of BAY 58-2667 to the enzyme (EC_{50} ODQ: 190 nM) (Stasch et al. 2006). The correlation of both EC_{50} values suggested that the oxidation of the prosthetic haem group of sGC resulted in the unmasking of a BAY 58-2667 binding site, leading to the observed increased binding to and activation of sGC. In contrast, haem-free sGC was directly activated more than 200-fold by BAY 58-2667 (Stasch et al. 2002; Schmidt et al. 2003) and this effect was not potentiated by the addition of ODQ. In the following years further investigations were able to solve this puzzle and reveal the nature of the BAY 58-2667 binding site.

Several lines of evidence suggest a model of BAY 58-2667-induced enzyme activation in which BAY 58-2667 binds to the sGC haem-pocket, forcing the enzyme into a conformation which resembles the NO-activated state (Fig. 3). sGC activity assays and binding studies revealed that PPIX and various metalloporphyrins (Zn-PPIX, Mn-PPIX) are capable of competing with the binding of BAY 58-2667 (Stasch et al. 2006). In addition, it was shown that the unique sGC-haem-binding motif Y-S-R (Pellicena et al. 2004; Schmidt et al. 2004, 2005) is crucially important for the interaction with the negatively charged groups of both the haem moiety and BAY 58-2667. The analogy between the haem moiety and BAY 58-2667 was further strengthened by structural alignments which shown that BAY 58-2667 is able to mimic the spatial structure of the sGC porphyrin ligand (Schmidt et al. 2004, Hahn et al. 2007).

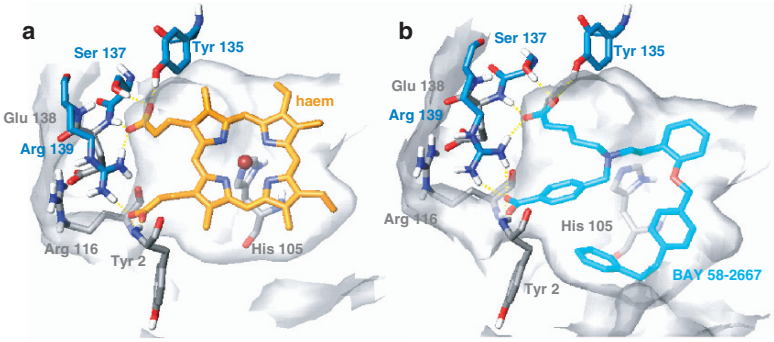

Fig. 3 Homology model of the haem binding domain of rat sGC. (**a**) Haem binding site; the Y–S–R motif is coloured blue. (**b**) Assumed binding mode of BAY 58-2667. The bound sGC activator mimics the haem group

Although this hypothesis easily explains the strong BAY 58-2667-induced activation of haem-free sGC via its binding to the orphaned haem-binding site, the precise mechanism which leads to the activation of haem-oxidized sGC is still under discussion. Schmidt and co-workers proposed a model in which BAY 58-2667 can replace the prosthetic group of the enzyme. This hypothesis was supported by the direct spectroscopic observation that BAY 58-2667 is able to replace even the tightly bound reduced haem at concentrations exceeding 30 μM (Schmidt et al. 2004). It is generally accepted that sGC haem oxidation dramatically reduces the affinity of the prosthetic group for the enzyme (Ignarro et al. 1984; Hobbs 2000; Roy et al. 2008). The reduced affinity of the haem would in turn strongly facilitate the replacement of the prosthetic group by BAY 58-2667 resulting in similar EC50 values to those determined for the haem-free sGC.

Nevertheless, very recently Roy and co-workers published compelling evidence that haem-independent sGC activators such as BAY 58-2667 or HMR 1766 target only the haem-free state of sGC whereas the oxidized enzyme was virtually unresponsive to both compounds. In agreement with published results, Roy and co-workers found that the oxidation of the prosthetic group by compounds such as ODQ indeed strongly weakens the binding of the prosthetic group to the enzyme. However, in contrast to the initially proposed active replacement of the weakly bound haem by BAY 58-2667, the authors argue that oxidation results in an increased spontaneous haem loss which in turn allows BAY 58-2667 to bind at the now unoccupied haem pocket. Although the results leading to this hypothesis are conclusive they cannot entirely explain the direct spectroscopic observation that BAY 58-2667 displaces not only the oxidized haem but at high concentrations also the tightly bound reduced prosthetic group. There is no controversy about the general mechanism of the BAY 58-2667-induced sGC activation; however, further research is needed to clarify whether BAY 58-2667 is able to replace the weakly bound haem, or whether oxidation-increased spontaneous haem loss is responsible for the observed potentiation of BAY 58-2667 by ODQ. Despite this question remains open, this chapter will use the term 'haem-free' to emphasize that BAY 58-2667 activates sGC via binding to the haem pocket independently of the mechanism causing loss of the prosthetic group.

In light of these findings, the initial observation that BAY 58-2667 activates purified enzyme preparations strongly indicates that virtually all sGC preparations contain to some extent a BAY 58-2667-sensitive, haem-free subpopulation of the enzyme, although the exact ratio is hard to determine (Stasch et al. 2006). Therefore, this compound has the capability to serve as an invaluable biochemical tool to determine the relative amount of haem-free sGC in purified enzyme preparations, cells or tissues (Gladwin 2006; Stasch et al. 2006; Rajendran and Chirkov 2008).

HMR1766/S3448

The first paper describing the sGC-activating properties of this novel structural class in detail was published by Schindler and co-workers (Fig. 2) (Schindler et al. 2005, 2006). As no further report has described the biochemical characteristics of this

structural class so far, only limited conclusions can be drawn with respect to the mechanisms underlying the HMR1766-induced enzyme activation. HMR1766 activated a purified sGC preparation concentration-dependently up to 50-fold. The determined EC_{50} values for HMR1766 and the chemically-related structure S3448 were 0.51 and 0.68 μM, respectively (Schindler et al. 2006). In contrast to BAY 41-2272 and related structures, addition of submaximal concentrations of NO to HMR1766 resulted in an additive effect over the whole range of concentration. Kinetic analysis of the HMR1766-induced sGC activation showed that the enzyme activation was triggered by an approximately tenfold increase in the maximal catalytic rate V_{max} accompanied by a slight decrease of the K_M value from 73 to 46 μM (Schindler et al. 2005, 2006). As expected, oxidation of the sGC haem moiety virtually abolished the NO-induced enzyme activation but strongly potentiated the effect of S3448. The sGC-activating haem-site-ligand PPIX acted as a competitive agonist by concentration-dependently inhibiting enzyme activation by HMR1766 and S3448, respectively. These results suggest that the binding site for HMR1766/S3448 overlaps at least in part with porphyrins like PPIX. This hypothesis is in agreement with the observation that Zn-PPIX, a high affinity haem pocket antagonist, abolished enzyme activation by HMR1766 and S3448. Schindler and co-workers claimed that, in contrast to BAY 58-2667, both compounds activate the oxidized haem-containing sGC, whereas the haem-deficient enzyme is unresponsive to HMR1766 or S3448. However, this hypothesis is not supported by the authors' data as a haem-free purified enzyme preparation (haem-deficiency was validated by the absence of the Soret peak in wavelength scans and by the inability of NO and YC-1 to activate the enzyme preparation) could be strongly activated by S3448. These results are in agreement with recent findings by Roy and co-workers (Roy et al. 2008) showing that HMR1766 strongly activates haem-free sGC, whereas the observed activation of the oxidized enzyme seems to depend on the oxidation-augmented spontaneous haem-loss. Although structurally unrelated, it is most likely that HMR1766 and S3448 activate sGC by a comparable mechanism to BAY 58-2667. In particular, the observation that Zn-PPIX is able to abolish the activating effect of HMR1766 indicates an interaction with the haem-binding pocket of the enzyme as shown for BAY 58-2667. However, despite the similarities in the general characteristics of BAY 58-2667 and HMR1766, there seems to be differences in the molecular mechanism of sGC activation, such as the interaction with the haem-binding motif Tyr–Ser–Arg (Y–S–R). Mutation of these residues, which are crucially involved in coordinating the carboxylic groups of both the haem moiety and BAY 58-2667, abolished haem-binding to sGC and, in parallel, shifted the EC_{50} for the BAY 58-2667-induced sGC activation up to four orders of magnitude (Schmidt et al. 2004, 2005). In contrast, the sGC-activating effect of HMR1766 was less vulnerable to these mutations (Hoffmann et al. 2008). A recent publication was able to show an increased response of HMR-1766 in cells exposed to different form of oxidative stress (Zhou et al. 2008). Pre-treatment of smooth muscle cells with H2O2 significantly increased the HMR-1766 responses, whereas cGMP production stimulated by sodium nitroprusside (SNP) was decreased. Similar results were obtained with 3-morpholinosydnonimine (SIN-1), menadione and rotenone. In addition, HMR-

1766 was more effective in stimulating heme-free sGC as compared to the wild-type enzyme. Interestingly, in cells expressing heme-free sGC H2O2 inhibited, instead of potentiating the HMR-1766 responses, suggesting that the ROS-induced enhancement of cGMP formation was heme-dependent. Moreover, using truncated forms of sGC, it was observed that the N-terminus of the beta1 subunit of sGC is required for the action of HMR-1766. Taken together, there is compelling evidence that HMR1766 as well as BAY 58-2667 are interacting with the sGC haem-binding pocket (Schmidt et al. 2004; Schindler et al. 2005; Roy et al. 2008). Both compounds show comparable characteristics with respect to enzyme activation, such as their capability to activate the haem-free state of the enzyme. However, whereas BAY 58-2667 seems to share some properties with the native haem, the molecular mechanisms by which HMR1766 activates sGC remain to be discovered.

3.1.3 Targeting Haem-Free sGC

Since the discovery of sGC as the major NO receptor and the subsequent identification of the mechanism of enzyme activation, it has been accepted that reduced NO-sensitive sGC is the physiological form of the enzyme. The NO-insensitive haem-free or haem-oxidized states of the enzyme were assumed to be an artefact presumably generated in the process of enzyme purification, and great efforts have been undertaken (e.g. supplementation of media with haem, haem-precursor, iron and reductants) to optimize the expression and purification of the reduced haem-containing enzyme (Hoenicka et al. 1999). The so-called artificial states of sGC have found wide application as experimental tools to investigate the mechanisms of enzyme activation (Wedel et al. 1994; Garthwaite et al. 1995; Zhao et al. 1998, 2000; Martin et al. 2001). However, it was not until the discovery of BAY 58-2667 and its ability to solely activate NO-insensitive states of sGC that the identification of even small amounts of haem-free sGC under physiological conditions has become possible, broadening our view on the existence of different sub-populations of sGC (Schmidt et al. 2004; Gladwin 2006; Stasch et al. 2006; Rajendran and Chirkov 2008). It is unquestionable that NO-sensitive reduced sGC is the major physiological form of the enzyme. However, genetic knockout studies have shown that a fractional amount of activated sGC is capable of inducing vasorelaxation (Mergia et al. 2006) suggesting that even small increases in the amount of oxidized/haem-free sGC might have a strong impact on the vasorelaxing effect of BAY 58-2667 (Roy et al. 2008).

By taking advantage of the ability of BAY 58-2667 to solely activate haem-free sGC, Stasch and co-workers were able to show that NO-insensitive sGC is ubiquitously present in cells, tissues, and in vivo under physiological conditions (Stasch et al. 2002a; Schmidt et al. 2004). These findings turned these supposedly artificial states of the enzyme into novel pharmacological targets, which can be selectively activated by compounds such as BAY 58-2667 or HMR1766.

In addition to basal cellular levels of haem-free sGC, it has been speculated that the amount of oxidized enzyme (which in turn is prone to haem loss) could be

elevated as a consequence of cardiovascular disorders, which are accompanied by increased levels of oxidative stress including the formation of superoxide and/or peroxynitrite (Weber et al. 2001; Francois and Kojda 2004). However, this hypothesis has been hard to test in the absence of compounds, which selectively activate haem-free sGC. By using BAY 58-2667 as a biochemical tool, Stasch and co-workers were able to show that increased levels of oxidative stress arising from vascular disease conditions markedly elevated the effect of BAY 58-2667 (Stasch et al. 2006). The in vivo relevance of these findings was substantiated by testing in a broad range of cardiovascular disease models, namely spontaneously hypertensive rats (SHR) as a model of hypertension; Watanabe heritable hyperlipidemic rabbits and ApoE –/– mice as models of atherosclerosis; and mesocolon arteries from patients with type 2 diabetes as a model for diabetes. The authors were able to show that BAY 58-2667 markedly improved relaxation in aortas of SHR as well as Watanabe rabbits compared with their respective control animals. The NO-donor nitroprusside was similarly effective in Watanabe and wild-type rabbits indicating that even minor increases in the amount of haem-free sGC might impact on the vasorelaxing effect of haem-independent sGC activators (Mergia et al. 2006). In agreement with these results, mesocolon arteries obtained from diabetic patients were more potently relaxed by BAY 58-2667 than arteries of nondiabetic patients. The observed ex vivo vasorelaxation was reflected by the blood pressure lowering effect of BAY 58-2667 after oral administration in SHR and transgenic renin rats treated with *N*-nitro-L-arginine-methylester to inhibit NO synthase (Stasch et al. 2006). Schindler and co-workers were able to show that the impact of the haem-indpendent sGC activator HMR1766 on the relaxation of preconstriced rat aortas as well as the reduction of blood pressure in piglets was markedly increased in the presence of the haem-oxidizing sGC inhibitor ODQ (Schindler et al. 2006). Taken together, the results published to date suggest that under pathophysiological conditions the ratio of haem-free/reduced sGC can be shifted to a BAY 58-2667/HMR1766-sensitive form, indicating that haem-independent sGC activators might be the first-known drugs which specifically target a cardiovascular disease state (Evgenov et al. 2006; Stasch et al. 2006; Zhou et al. 2008).

In addition to the published work, which suggests the existence of basal levels of oxidation-induced haem-free sGC under physiological conditions, recent findings indicate the existence of a further source of haem-free sGC in living cells. It has been shown that induction of haem-oxygenase 1 (HO-1) in endothelial cells or vessels by $CoCl_2$ or by retroviral gene transfer results in the depletion of cellular haem levels and, as a consequence, in a reduced generation of cGMP upon incubation with NO donors (Abraham et al. 2002; Mingone et al. 2008). The fact that haem-free sGC might originate from increased degradation of the prosthetic group indicated the presence of haem-free sGC in living cells. These findings have been strengthened by recent results which show that addition of the haem-precursor δ-aminolevulinate to the medium resulted in the increased formation of cellular PPIX and most importantly in a concentration-dependent increase of cellular cGMP levels (Mingone et al. 2006). As cellular levels of sGC did not increase, the observed cGMP formation has to be in the wake of the reconstitution of the enzyme with PPIX (Mingone

et al. 2006). However, as the affinity of PPIX for sGC is rather low it seems unlikely that the concentration of additionally synthesized PPIX is high enough to displace the haem from the enzyme. Therefore, the results by Mingone and co-workers could be taken as indications for the existence of a further source of haem-free sGC which seems to be regulated by the balance of haem synthesis and haem catabolism.

3.1.4 Protection of sGC Against Degradation

Like any protein sGC is subject to physiological cellular turnover. Although the half-life of sGC was shown to be approximately 7 hours (Xia et al. 2007), there are several factors which influence the stability of the enzyme. It was reported that sGC, by virtue of its association with a multimeric protein complex including HSP90, HSP70 and eNOS (Venema et al. 2003; Balashova et al. 2005; Antonova et al. 2007), is sensitive to the HSP90 inhibitor geldanamycin, resulting in a decrease of sGC protein levels (Papapetropoulos et al. 2005; Nedvetsky et al. 2008). Very recent findings showed that this decrease is mediated via the ubiquitinylation of sGC and its subsequent degradation via the ubiquitin–proteasome-pathway (Xia et al. 2007; Meurer et al. 2008). Within this pathway, the E_3 ligase CHIP, which interacts with the sGC/HSP90/HSP70 protein complex, was identified as one of the key players in mediating the ubiquitinylation of sGC (Xia et al. 2007; Meurer et al. 2008).

Beside the reduction of sGC protein levels by HSP90 inhibitors, it has been known that cardiovascular diseases which result in increased levels of oxidative stress in the vessel wall are accompanied by a reduced expression of sGC (Bauersachs et al. 1998; Ruetten et al. 1999). In agreement with these findings, Stasch and co-workers were able to show that oxidation of the prosthetic sGC haem moiety to its NO-insensitive Fe^{3+} form results in a substantial reduction of sGC protein levels (Stasch et al. 2006). Later investigations supported these initial findings and were able to show that the observed oxidation-induced sGC-degradation is caused by a CHIP-mediated ubiquitinylation of sGC (Meurer et al. 2008). Although the exact molecular mechanism by which oxidation of sGC triggers its ubiquitin-mediated degradation remains to be clarified, it is evident that the nature of the haem-site ligand as well as its oxidation state is intimately involved in this mechanism (Fig. 4).

Recent publications were able to show that the NO-independent sGC activator, BAY 58-2667, is not only able to prolong significantly the physiological half-life of sGC, but also to completely reverse the oxidation-induced degradation of sGC, increasing sGC protein levels beyond control levels (Stasch et al. 2006; Meurer et al. 2008). Other compounds which increase cellular cGMP levels such as NO, PDE5 inhibitors, 8-Br-cGMP, YC-1 and BAY 41-2272 did not increase sGC protein levels, ruling out the hypothesis that the observed effect of BAY 58-2667 is due to generated cGMP (Stasch et al. 2006; Hoffmann et al. 2008).

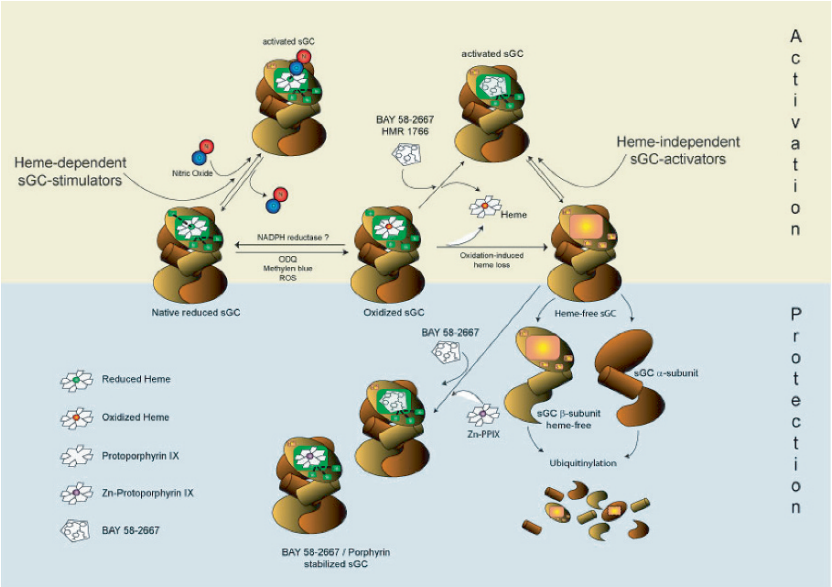

Fig. 4 The NO receptor sGC exists in a physiological equilibrium between two redox states; the native reduced and NO-sensitive form (*left*) can be oxidized by oxidants like ODQ, ROS or methylene blue. This leads to an oxidation-induced loss of the redox-sensitive prosthetic haem group. The haem-free sGC undergoes ubiquitin-mediated proteasomal degradation as shown previously (Stasch et al. 2006, Meurer et al. 2008). Under pathological conditions, the redox equilibrium is shifted towards the oxidation-induced haem-free form, which is selectively targeted by sGC activators like BAY 58-2667. In addition, high-affinity haem-site ligands such as Zn-PPIX and BAY 58-2667 stabilize the haem-free β1 subunits by binding to the orphaned haem-binding site. In turn, the prevention of sGC β1 degradation presumably results in the indirect stabilisation of the alpha subunit

Although the exact nature of this significant sGC stabilization by BAY 58-2667 has not been established yet, several lines of evidence suggest a hypothesis for the mechanism of protein stabilization. Schmidt and co-workers were able to show that the porphyrin-mimic BAY 58-2667 strongly activates haem-free sGC by directly binding to the orphaned haem-pocket (Schmidt et al. 2004). Due to its mechanism of enzyme activation it seems likely that binding of an oxidation-resistant high-affinity haem-site ligand such as BAY 58-2667 to the sGC haem-pocket leads to the observed stabilization of the enzyme and, as a consequence, to the increased sGC protein levels. This hypothesis is supported by the fact that zinc-protoporphyrin IX (Zn-PPIX), which is not affected by oxidation and binds to the sGC haem-pocket with nanomolar affinity, is able to mimic the BAY 58-2667-induced sGC stabilization (Stasch et al. 2006; Meurer et al. 2008). Interestingly, the sGC activator HMR1766, which shares many characteristics with BAY 58-2667 with respect to enzyme activation, was not able to stabilize sGC protein levels, underlining that the mechanism of action of this compound is presumably not via mimicking the spatial structure of porphyrins (Hoffmann et al. 2008).

Taken together, the published data indicate that high affinity haem-site ligands are capable of counter-regulating the ubiquitin-mediated degradation of oxidation-impaired sGC and thereby increasing the enzyme's protein levels. However, whereas the sGC antagonist Zn-PPIX presumably results in an accumulation of inactive enzyme, BAY 58-2667 has theoretically the unique ability not only to activate oxidation-impaired NO-insensitive sGC but also to stabilize sGC in an activated state resulting in a kind of positive feedback loop.

4 Pharmacological Actions of sGC Activators

4.1 Arterial Hypertension

The dicarboxylic class of sGC activators comprises potent vasodilators such as BAY 58-2667 and BAY 60-2770, which induce systemic blood pressure lowering (Stasch et al. 2002a; Knorr et al. 2008). In the case of BAY 58-2667, the potency and duration of this dose-dependent and long-lasting blood pressure lowering in anaesthetised rats was significantly increased in potency and duration by pre-treatment with the oxidative sGC inhibitor ODQ (Stasch et al. 2006). Importantly, these data suggest that unlike nitroglycerin, the in vivo efficacy of BAY 58-2667 is not reduced by oxidative stress but rather augmented due to targeting of the haem-free sGC. Due to its distinctly lower potency, HMR1766 did not show any direct blood pressure lowering effect in humans or animals (Schindler et al. 2005; Oberwittler et al. 2007).

There is evidence that sGC activators show therapeutic effects in the cardiovascular system beyond blood pressure lowering. Benz et al. (2007) and Jones et al. (2008) observed beneficial effects of HMR1766 and BAY 58-2667 in chronic renal disease and hypertension-induced cardiac hypertrophy that were independent of relevant blood pressure lowering. Thus the possibility arises that sGC activators show anti-remodelling effects in cardiovacular disease, as has already been shown in different models of PH (Dumitrascu et al. 2006).

4.2 Pulmonary Hypertension (PH)

The pulmonary circulation is regulated differently to systemic blood vessels, and the two systems may therefore differ in terms of sensitivity to sGC activators (Coggins and Bloch 2007). A hallmark of this regional specificity is the profound difference in response to hypoxia: vasoconstriction in the lung and vasodilatation systemically. Moreover, NO is critical for the normal development of the pulmonary vasculature and vasoregulation in the adult. It is generated both in the vascular endothelium and airway epithelium of the lung, adjusting the vascular perfusion to the respective alveolar ventilation. Reduced NO biosynthesis or bioavailability may explain

endothelial dysfunction and vascular pathology in PH, a life-threatening condition characterized by increased pulmonary arterial pressure due to elevated pulmonary vascular resistance. Chronically, PH is associated with pulmonary vascular remodelling, localized thrombosis and right heart hypertrophy (Farber and Loscalzo 2004; Evgenov et al. 2006). Treatment of PH largely remains palliative rather than curative. Inhaled NO may be therapeutically active by substituting for insufficient endogenous NO to produce pulmonary vasodilation in well-ventilated lung regions without causing systemic arterial hypotension (Ichinose et al. 2004). Inhalation of gaseous NO is used to treat PH of various etiologies, but NO has a very short duration of action, leads to methaemoglobinemia, and is ineffective in some PH patients (Ichinose et al. 2004; Bloch et al. 2007). Since the pulmonary vasodilation of NO is mostly mediated by cGMP, inhibition of the cGMP-metabolizing phosphodiesterases (PDEs) has a similar effect. For example, oral or intravenous administration of the PDE5 inhibitor sildenafil produces pulmonary vasodilation in experimental models of PH, as well as in patients (Ghofrani et al. 2006). Furthermore, sildenafil is able to augment the pulmonary vasodilator response to inhaled NO (Stocker et al. 2003). However, a significant proportion of the patients with PH also fail to respond to sildenafil, indicating that endogenous NO production in these patients is reduced to such an extent that inhibition of cGMP degradation has no beneficial effects (Weimann et al. 2000) or that NO-cGMP signalling is not primarily affected (Kirsch et al. 2008).

In a hypoxic mouse model of acute, sub-acute and chronic PH, cGMP and cGMP signalling were in fact unchanged (Kirsch et al. 2008), and sildenafil increased cGMP to supraphysiological levels. Thus sildenafil appears to act in a symptomatic manner and not by correcting a cGMP-related pathomechanism. In this study, total sGC levels were not affected; however, haem-free sGC content and responsiveness were not investigated and could be elevated even when total sGC levels are normal. Thus, activation of sGC may still represent a promising therapeutic approach in PH.

Indeed, in mice with chronic hypoxia-induced PH and rats with chronic monocrotaline-induced PH, oral treatment with BAY 58-2667 markedly reduced right ventricular systolic pressure. This beneficial hemodynamic effect was associated with an improvement of right ventricular hypertrophy and structural remodeling of the lung vasculature as reflected by reduced muscularization of peripheral pulmonary arteries (Dumitrascu et al. 2006). Notably, treatment with this agent was commenced after full establishment of PH, right heart hypertrophy, and structural changes in the lung vasculature, suggesting that BAY 58-2667 reversed progression of the disease rather than simply preventing it.

PH can be associated with idiopathic pulmonary fibrosis, in which repeated microscopic injury and failed repair of the alveolar epithelium cause proliferation of interstitial fibroblasts, leading to the development of fibroblast/myofibroblast foci and lung fibrosis (Patel et al. 2007). Lung tissue remodelling and fibrosis also occur in chronic obstructive pulmonary disease (COPD). BAY 58-2667 may have dual beneficial effects in PH associated with lung fibrosis; it was shown to prevent the conversion of human lung fibroblasts into myofibroblasts as well as relieving PH by promoting vasodilatation (Dunkern et al. 2007). Tissue factor (TF), expressed by

circulating blood monocytes and vascular endothelium, is also upregulated in the pulmonary vasculature in PH. BAY 58-2667 attenuated TF protein expression and function in human monocytes. This effect was associated with a marked reduction of TF surface presentation in monocytes. BAY 58-2667 also reduced total and surface TF protein levels in human umbilical vein endothelial cells (HUVEC), followed by a reduction of TF-dependent procoagulant activity in the lysates of HUVEC. These findings suggest that inhibition of TF expression and activity by sGC activators might provide therapeutic benefits in PH and other cardiovascular diseases (Evgenov et al. 2008).

As observed with BAY 58-2667, HMR1766 attenuated the development of PH and right heart hypertrophy, preserved systemic arterial blood pressure and improved gas exchange and cardiac output in monocrotaline-induced PH (Schäfer and Bauersachs 2007). In a model of transforming growth factor-β (TGF-β)-induced cardiac fibroblast extracellular matrix synthesis, HMR1766 inhibited fibronectin protein synthesis in a concentration-dependent manner and prevented the differentiation of fibroblasts into myofibroblasts (Illiano et al. 2006).

Treatment of PH with orally or intravenously administered vasodilating drugs including NO-releasing drugs, prostacyclin analogs and PDE inhibitors, as well as agonists of sGC, can be associated with potentially unwanted systemic hypotension and impairment of pulmonary gas exchange (Paton and Byron 2007; Gessler et al. 2002). In contrast, targeted drug delivery to the lungs via inhalation can result in a rapid onset of action, high local bioavailability and low metabolism, potentially avoiding or reducing systemic side effects. Indeed, inhalation of biodegradable, dry-powder microparticles containing the sGC activator BAY 58-2667 produced potent selective pulmonary vasodilation and increased transpulmonary cGMP release without significant effect on mean arterial pressure in an ovine model of acute PH (Evgenov et al. 2007). Intravenous administration of ODQ, which oxidizes the prosthetic haem group of sGC, markedly reduced the pulmonary vasodilator effect of inhaled NO. In contrast, pulmonary vasodilation and transpulmonary cGMP release induced by inhaling BAY 58-2667 microparticles were greatly enhanced after treatment with ODQ. Therefore, inhalation of microparticles containing an sGC activator may provide an effective novel treatment for patients with PH, particularly when responsiveness to inhaled NO is impaired under oxidative stress conditions known to be associated with cardiopulmonary diseases (Chirkov and Horowitz 2007; Coggins and Bloch 2007; Evgenov et al. 2007).

4.3 Peripheral Arterial Disease

The main characteristic of stage II peripheral arterial occlusive disease (PAOD) is exercise-induced muscle fatigue. The effect of HMR1766 has been studied in Zucker Diabetic Fatty (ZDF) rats with unilateral hind limb ischemia as an experimental model of PAOD. Placebo-treated animals showed marked signs of

exercise-induced muscle fatigue, with a mildly reduced contraction force, moderately reduced contraction velocity, severely reduced relaxation velocity and markedly prolonged time to recovery after contractions. All of these parameters significantly improved in animals treated with HMR1766 (Schäfer and Bauersachs 2007). HMR1766 is currently in phase II clinical development for intermittent claudication in patients with Fontaine stage II peripheral artery disease (ACCELA trial). It is possible that sGC activators exert a predominantly microvascular effect that can be exploited under these conditions.

4.4 Heart Failure

Chronic heart failure (CHF) is the major cause of death and morbidity worldwide and a growing public health issue. Mechanistically, several organs and different cell types are involved. The underlying structural and functional abnormalities affect not only the heart but also blood vessels, sodium homoestasis and neurohumoral regulation. The progression from cardiovascular dysfunction to heart failure involves numerous signalling pathways (Hare and Stamler 2005; Pacher et al. 2007). With respect to cGMP, both NO formation and bioavailability as well as natriuretic peptide release are affected, resulting in an impairment of both the sGC and particulate (p)GC pathways (Boerrigter and Burnett 2004; Pacher et al. 2007). Organic nitrates have been used for more than a century to treat cardiovascular diseases. However, their efficacy is limited because of tolerance development, most likely due to incomplete bioactivation to NO. Thus, pGC or sGC activators that do not rely on such bioactivation mechanisms have potential as long-term therapies to elevate cGMP in heart failure, which may open entirely new therapeutic options and drug combinations.

Within the group of sGC activators, only BAY 58-2667 seems to have been studied clinically for this indication. Multiple sites of action may contribute to its efficacy, none of them necessarily in the heart. In contrast, this compound class has potent cardiac unloading actions both in normal dogs and in experimental heart failure. In the first case, intravenous administration of BAY 58-2667 dose-dependently decreased mean arterial, right atrial, diastolic pulmonary arterial, central venous and left ventricular end-diastolic pressure (Boerrigter et al. 2007a; Hoffmann and Thuss 2007). Nitroglycerin produced similar haemodynamic responses; however, the duration of its vasodilator effects was much shorter (Stasch et al. 2002a). The cardiorenal actions of BAY 58-2667 were investigated in a canine model of congestive heart failure (CHF), induced by rapid ventricular pacing for 10 days (Boerrigter et al. 2007a). BAY 58-2667 potently reduced cardiac pre- and after-load in a dose-dependent manner, as indicated by decreases in mean arterial, right atrial, pulmonary arterial and pulmonary capillary wedge pressure caused by a cGMP-mediated vasorelaxation of both veins and arteries. Concomitant with the decrease in systemic and renal vascular resistance, there were increases in cardiac output and renal blood flow.

Importantly, renal function was maintained despite the reduction in mean arterial pressure. There was no activation of the renin–angiotensin–aldosterone system, and an observed reduction in natriuretic peptide levels can be interpreted as a neurohumoral marker for beneficial cardiac unloading (Boeringter et al. 2007a).

With respect to combination therapy, co-administration of BAY 58-2667 and BNP combines the haemodynamic actions of BAY 58-2667 with the renal enhancing properties of BNP, suggesting that co-activation of soluble GC and pGC may also be a beneficial strategy for the treatment of ADHF, optimizing activation of distinct GC enzymes and cGMP synthesis (Boerrigter et al. 2007b).

Moreover, beneficial effects of chronic activation of sGC on cardiac remodelling have been observed in aged SHR (Jones et al. 2008). Importantly, these anti-hypertrophic and anti-fibrotic effects occurred in spite of maintained hypertension, suggesting the involvement of a blood pressure-independent mechanism in the cardioprotective effects of direct sGC activation.

Compared with nitrate therapy, sGC activators such as BAY 58-2667 seem to have several advantages. These agents preferentially and very effectively activate sGC when it is in the oxidized or haem-free state, promote vasodilatation in vessels where endogenous NO fails to function, and thereby show beneficial effects under conditions of endothelial dysfunction (Stasch et al. 2006; Gladwin 2006). They do not promote oxidative stress, as do organic nitrates. Moreover, they do not need to be bioactivated like organic nitrates, and, thus, impairments to organic nitrate activation caused by oxidant stress would not alter their effectiveness. Taken together, these direct sGC activators would seem to avoid many of the untoward effects of long-term nitrate therapy and represent an entirely new pharmacological principle that selectively targets diseased blood vessels (Stasch et al. 2006; Chirkov and Horowitz 2007; Münzel et al. 2007; Ritz 2007).

4.5 Anti-Platelet Effects

Endogenously, endothelium-derived NO is one of the mechanisms by which platelet aggregation and thrombus formation is prevented by the intact blood vessel wall. Pharmacological stimulation of sGC in platelets correlates with inhibition of aggregation, platelet cGMP increase, prolongation of bleeding time and antithrombotic effects in vitro (Hobbs 2000; Stasch et al. 2002a, b, c, Tulis 2007; Moncada and Higgs 2006). This protective mechanism may be impaired in vascular disease and impaired NO availability. In addition, haem-free sGC may also be present in platelets and levels may be increased under disease conditions, similar to the situation in blood vessel walls (Roy et al. 2008). Thus sGC activators may have antithrombotic effects similar to endogenous NO. Indeed, BAY 58-2667 potently inhibited platelet aggregation induced by the thromboxane mimic U46619 and collagen, whereas TRAP-6- and thrombin-mediated aggregation was not affected. In human platelets, BAY 58-2667 increased both cGMP levels and cGMP-dependent protein kinase-mediated vasodilator-stimulated phosphoprotein (VASP) phosphorylation,

as did the NO donor sodium nitroprusside. Moreover, an up to twofold prolongation in rat-tail bleeding time was also observed after oral administration of BAY 58-2667. More importantly, BAY 58-2667 reduced thrombus formation in the $FeCl_3$ thrombosis rat model. Oral BAY 58-2667 induced a dose-dependent increase in platelet cGMP content and VASP phosphorylation, which may serve as a biomarker for its efficacy.

The use of BAY 58-2667 as a tool for probing the haem occupancy of GC in cells indicated that in rat platelets only a very small proportion of enzyme normally exists in the haem-free state. However, the proportion of haem-free enzyme in platelets increased distinctly under conditions of haem oxidation presumably due to increased spontaneous haem-loss (Roy et al. 2008).

This event, or a deficiency in the incorporation of haem into the protein, could account for the enhanced potency of BAY 58-2667 observed in spontaneous and experimental pathologies (Dumitrascu et al. 2006; Stasch et al. 2006; Boerrigter et al. 2007).

Thrombus formation in a coronary artery is the acute event in most unstable ischemic coronary syndromes. HMR1766, which induced no hemodynamic effects, showed beneficial effects in the Folts model of coronary thrombosis (Van Eickels et al. 2007). In contrast to aspirin, the effect of HMR1766 was not abolished by epinephrine, suggesting that HMR1766 will be effective under conditions of increased sympathetic tone (Van Eickels et al. 2007).

Diabetes mellitus is associated with an increased thromboembolic risk via activation of circulating platelets as well as the development of endothelial and vascular dysfunction. NO bioavailability and platelet sGC sensitivity towards NO is significantly decreased in diabetes. Chronic treatment with HMR1766 enhanced NO/cGMP-mediated signalling in platelets from diabetic rats, determined by in vivo phosphorylation of platelet VASP. In parallel, platelet-binding of fibrinogen, surface-expression of P-selectin and appearance of platelet-derived microparticles and platelet aggregates with other blood cells were significantly reduced by chronic treatment with HMR1766. Chronic activation of sGC by HMR1766 normalized vascular function and restored endothelium-dependent relaxation in isolated aortic rings from control and placebo-treated streptozotocin rats (Schäfer et al. 2006; Schäfer and Bauersachs 2007; Van Eickels et al. 2007). Taken together, these studies demonstrated that chronic activation of sGC in diabetic rats improves markers of platelet activation and therefore represents a viable approach for the prevention of cardiovascular complications in diabetes (Schäfer et al. 2006; Schäfer and Bauersachs 2007).

4.6 Anti-Atherosclerotic, Anti-Fibrotic Anti-Proliferative Effects

Atherosclerosis is associated with both decreased NO bioavailability and altered signal-transduction downstream of NO including neointimal sGC (Melichar et al. 2004; Tulis 2007). Cyclic GMP has not only acute effects but also chronic protective

effects on the vasculature, such as inhibition of smooth muscle cell proliferation, growth and migration. Metabolic tolerance and reflex tachycardia have precluded the long-term use of NO donors to treat atherosclerosis. However, sGC, and in particular haem-free sGC which is increased in diseased blood vessels, may represent an attractive novel pharmacological target in the long-term treatment of atherosclerosis and restenosis. Again, the blood pressure-independent low-dose anti-fibrotic effects of sGC activators may be at least partially responsible for their anti-atherosclerotic properties. In a mouse model of atherosclerosis (apolipoprotein E-deficient [ApoE–/–] mice fed a high-fat high-cholesterol diet), HMR1766 significantly reduced atherosclerotic plaque formation and markedly improved endothelium-dependent vasodilatation, without having any effect on endothelium-independent vasorelaxation or vasoconstriction. Aortic release of reactive oxygen species was still increased in ApoE–/– mice compared with wild-type mice and was not affected by the treatment. HMR1766 led to increased levels of vascular P-VASP, a downstream target of sGC and cGK, did not alter eNOS and sGC expression, and reduced vascular VCAM-1 expression (Schäfer and Bauersachs 2007).

Myocardial injury can trigger cardiac fibroblast proliferation, transformation into myofibroblasts and enhanced extracellular matrix production. The resulting cardiac fibrosis contributes to structural remodeling and ventricular dysfunction leading to heart failure. Natriuretic peptides, which stimulate membrane-bound GC to produce a subsequent rise in cGMP, have been reported to inhibit cardiac fibrosis, similar to activators and stimulators of sGC (Boerrigter and Burnett 2004; Lee and Burnett 2007). HMR1766 inhibits overall matrix synthesis induced by TGFß in cardiac fibroblasts and inhibits the differentiation of fibroblasts into myofibroblasts in vitro. Thus, the haemodynamic properties of HMR1766 may be complemented by an additional beneficial effect on cardiac fibrosis (Illiano et al. 2006).

Liver fibrosis and cirrhosis are late complications common to liver diseases of different etiology such as viral hepatitis and alcoholic liver disease. Irrespective of the initial cause of liver disease, activation of hepatic stellate cells is a crucial step in the fibrotic pathomechanism (Friedman 2004; Reynaert et al. 2002; Gäbele et al. 2003). Activation of hepatic stellate cells is reduced by an increase in cGMP (Kawada et al. 1996). However, cGMP production is downregulated in the cirrhotic liver due to reduced activity of the endothelial NO synthase and increased expression of PDE5 (Van de Casteele et al. 2002; Perri et al. 2006). Oral treatment with the sGC activator BAY 60-2770 in rat models of liver fibrosis (a pig serum model, a carbon tetrachloride model and an accelerated model that combines both stimuli) in doses not affecting systemic blood pressure prevented the increase in hepatic fibrous collagen and total collagen accumulation (Knorr et al. 2008). Therefore, direct activation of sGC might also provide a novel approach for the treatment of liver fibrosis of necro-inflammatory and immunological origin.

Finally, untreated chronic renal disease is characterized by a continuous decline in renal function. However, some renal diseases such as early stage diabetic nephropathy show an increased glomerular filtration rate (GFR). End-stage renal failure necessitates renal replacement therapy by either dialysis or a kidney transplant. Ongoing matrix protein expansion (i.e. renal fibrosis) is a hallmark of this

chronic disease process irrespective of whether the underlying process is hypertension, diabetes or autoimmune inflammation. When renal function becomes compromised beyond a certain point, fibrosis will proceed regardless of the activity of the original trigger. At the histological and molecular level, fibrotic renal disease is characterized by the accumulation of extracellular matrix proteins, such as collagens and fibronectin, and by the loss of specialized renal cells. The incidence of renal failure is rising worldwide due to the diabetes pandemic, obesity and hypertension, and the increasing age of hypertensive patients with cardiovascular disease, who now live long enough to develop renal disease. Therefore, delaying, or even preventing, the loss of renal function that leads to end-stage renal failure is the most important goal in the pharmacological management of chronic renal disease. Based on the efficacy of sGC stimulators in preventing matrix accumulation and tissue injury (Peters et al. 2004; Hohenstein et al. 2005; Wang et al. 2005 and 2006; Wang-Rosenke et al. 2008), recent studies evaluated the effects of long-term sGC activation with BAY 58-2667 on renal disease in models of chronic renal failure. In rats with subtotal nephrectomy, chronic treatment with BAY 58-2667 prevented the increase in blood pressure, preserved renal function, improved plasma levels of natriuretic peptides and reduced left ventricular hypertrophy and cardiac arterial wall thickness, as well as slowing renal disease progression (Kalk et al. 2006). The long-term renoprotective effects of a nonhypotensive dose of the sGC activator, HMR1766, were studied in the remnant kidney model, a noninflammatory rat model of renal failure; similar to BAY 58-2667, HMR1766 showed beneficial blood pressure-independent effects on kidney structure and urinary albumin excretion (Benz et al. 2007). These preclinical studies may argue for a novel therapeutic approach for the management of patients with chronic renal disease. Interestingly, the antifibrotic effect seems to occur at lower doses of sGC activator or is more sustainable than direct vascular and blood pressure lowering effects.

4.7 Protection of ischemic myocardium

Reperfusion therapy is the treatment of choice for acute myocardial infarction. Unfortunately it can seldom be instituted quickly enough to prevent some infarction in these patients. Approximately 25% of these patients have enough infarction to adversely affect pump function leading to remodeling and heart failure (Miura and Miki 2008). One approach to this problem would be to treat these heart at the time of reperfusion with an intervention that makes the heart resistant to infarction. Although several methods for doing this have been explored, by far the most promising has been ischemic preconditioning in which a brief ischemic period followed by reperfusion prior to a prolonged ischemic insult causes the heart to quickly adapt itself to become very resistant to infarction. The mechanism involves occupation of surface receptors to adenosine, bradykinin and opioid which are released during the transient ischemic period. These receptors ultimately activate protective kinases including ERK, Akt, and GSK3-beta which prevent formation of lethal permeabil-

ity transition pores in the heart's mitochondria when the heart is reperfused (Yellon and Downey 2003). Because preconditioning exerts if protection in the first minutes of reperfusion some agents can activate the preconditioning mechanism when administered just prior to reperfusion which would be a clinically feasible protocol.

A key step in the signal transduction pathway that couples the surface receptors to the protective kinases is activation of PKG by cGMP (Burley et al. 2007; Costa et al. 2005). Production of cGMP by natriuretic peptides just prior to therapeutic reperfusion has a significant anti-infarct effect in both animals (Yang et al. 2006) and humans (Kitakaze et al. 2007). The cell permeant cGMP analog 8-(4-chlorophenylthio)-guanosine 3′, 5′-cyclic monophosphate (CPT) is very protective when given to isolated hearts just prior to reperfusion (Kuno et al. 2008). The PDE5 inhibitor sildenafil has also been reported to prevent infarction in animal models (Salloum et al. 2007, 2008). BAY 58-2667 was tested in both isolated and in situ rabbit hearts as well as isolated rat hearts all exposed to 30 min of regional ischemia (Frenzel et al. 2008; Krieg et al. 2008). In that study BAY 58-2667 was administered for one hour starting just prior to reperfusion and as expected was equipotent with ischemic preconditioning at concentrations as low as 1 nM in the Krebs buffer-perfused isolated rabbit hearts. The drug was also tested in an open-chest rabbit model given as a loading dose 5 min prior to reperfusion followed by an infusion. The lower infusion dose that was tested had a insignificant effect on blood pressure but still reduced infarct size by approx. 50% (Krieg et al. 2008). A similar protection was seen in isolated rat hearts. Blocking studies confirmed that the mechanism included PKG and mitochondria ATP-sensitive potassium channels.

5 Clinical Trials of NO-Independent sGC Activators

A phase I clinical trial assessed the safety, tolerability and pharmacokinetics of ascending doses of intravenously administered BAY 58-2667 in healthy male volunteers (Frey et al. 2008). BAY 58-2667 exhibited dose-proportional pharmacokinetics for doses of 50–250 μg/h given intravenously for up to 4 h. In parallel, BAY 58-2667 had dose-dependent haemodynamic effects on diastolic blood pressure, mean arterial blood pressure and heart rate. BAY 58-2667 had potent preload- and afterload-reducing cardiovascular effects, a favourable safety profile and was well tolerated in healthy subjects (Frey et al. 2008).

A non-randomized, uncontrolled, unblinded, multicentre phase II study in patients with ADHF was performed (Lapp et al. 2007). After initial dose-finding studies using 50, 100, 200 and 400 μg/h, respectively, BAY 58-2667 was evaluated using a starting dose of 100 μg/h, which could be titrated after 2, 4 and 6 h to doses between 50 and 400 μg/h, depending on the haemodynamic response. In patients with ADHF, continuous infusion of BAY 58-2667 was well tolerated and resulted in potent arterial vasodilation, which was associated with a decrease in pulmonary capillary wedge pressure, right atrial pressure, systemic vascular resistance and pulmonary vascular resistance, and an increase in cardiac output. The proportion of

patients responding to BAY 58-2667 was 90% after 6 h. These results suggest that there is a pool of oxidized and/or haem-free sGC in humans with ADHF that is preferentially activated by BAY 58-2667. There was no evidence of tachyphylaxis. These first clinical results with BAY 58-2667 in ADHF patients demonstrate the potential of this new therapeutic principle; further investigation is therefore warranted (Lapp et al. 2007). A phase IIb clinical study in patients with ADHF is currently underway.

HMR1766 is in phase II clinical development for the treatment of PAOD. A randomized, double-blind, placebo-controlled dose-ranging trial of HMR1766 in patients with PAOD is currently underway, assessing the efficacy and safety of HMR1766 versus placebo (administered for 26 weeks), with cilostazol as a calibrator (ACCELA trial). A significant fraction of patients to be treated with HMR1766 is expected to be maintained on warfarin. Because HMR1766 has been shown to inhibit the warfarin-metabolizing enzyme CYP2C9, the effect of HMR1766 on warfarin pharmacokinetics and pharmacodynamics has been studied. A CYP2C9-mediated pharmacokinetic interaction, with pharmacodynamic, clinically relevant consequences which might require warfarin dose adjustment, was demonstrated (Oberwittler et al. 2007).

6 Future Directions

The next years will yield exciting data on the clinical potential of sGC activators; basic science is almost lagging behind, explaining what drug discovery and development has achieved. The field of sGC is slowly coming to grips with the high speed at which haem-free and other variants change our understanding of this important enzyme. Two major questions need to be addressed: what causes the formation of haem-free sGC, and what is its distribution?

In the introduction, we already mentioned that it is unclear whether haem-free sGC is consequence of oxidation, or defective haem biosynthesis/incorporation, or whether there is a regulated pathway leading to its occurrence. In that case, should we expect to find endogenous activators of haem-free sGC? The example of HMR1766 shows that such a compound does not necessarily have to be a perfect haem mimic such as BAY 58-2667. Given the wide distribution of NO-cGMP signalling, it will be of interest to define the localisation and expression levels of oxidized/haem-free sGC using tools other than the bioassay of BAY 58-2667-induced cGMP elevation. Such reagents may evolve from fluorescent haem-site ligands or biophysical methods that detect the redox state of sGC's haem in intact cells and tissues, and may also include plasma-based assays and new biomarkers to identify patients with high levels of haem-free sGC. With respect to pathophysiology, are cardiovascular diseases the only state in which (Fe^{2+}) sGC is diminished and haem-free sGC increased? One therapeutic target may have multiple indications, also termed the "common mechanism strategy" in drug discovery (Nielsch et al. 2008). New indications (Chirkov and Horowitz 2007; Lincoln 2004) that were

inaccessible to previous therapies due to NO donor tolerance and NO resistance phenomena can now be tested (Jackson et al. 2007). In particular, the effect of sGC activators on blood vessel resistance may open up new avenues for the prevention of end-organ damage in the retina, kidney and diabetes. Stroke, migraine and Raynaud syndrome may also be viable targets; combinations of sGC and pGC activators and PDE5 inhibitors may increase efficacy in many classical cardiovascular indications. The race between basic and clinical science and drug discovery is still on.

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Natriuretic Peptides: Their Structures, Receptors, Physiologic Functions and Therapeutic Applications

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Abstract Natriuretic peptides are a family of three structurally related hormone/paracrine factors. Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are secreted from the cardiac atria and ventricles, respectively. ANP signals in an endocrine and paracrine manner to decrease blood pressure and cardiac hypertrophy. BNP acts locally to reduce ventricular fibrosis. C-type natriuretic peptide (CNP) primarily stimulates long bone growth but likely serves unappreciated functions as well. ANP and BNP activate the transmembrane guanylyl cyclase, natriuretic peptide receptor-A (NPR-A). CNP activates a related cyclase, natriuretic

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peptide receptor-B (NPR-B). Both receptors catalyze the synthesis of cGMP, which mediates most known effects of natriuretic peptides. A third natriuretic peptide receptor, natriuretic peptide receptor-C (NPR-C), clears natriuretic peptides from the circulation through receptor-mediated internalization and degradation. However, a signaling function for the receptor has been suggested as well. Targeted disruptions of the genes encoding all natriuretic peptides and their receptors have been generated in mice, which display unique physiologies. A few mutations in these proteins have been reported in humans. Synthetic analogs of ANP (anaritide and carperitide) and BNP (nesiritide) have been investigated as potential therapies for the treatment of decompensated heart failure and other diseases. Anaritide and nesiritide are approved for use in acute decompensated heart failure, but recent studies have cast doubt on their safety and effectiveness. New clinical trials are examining the effect of nesiritide and novel peptides, like CD-NP, on these critical parameters. In this review, the history, structure, function, and clinical applications of natriuretic peptides and their receptors are discussed.

1 History of Natriuretic Peptides

Key cell biological observations that predicted the existence of natriuretic peptides were reported over fifty years ago. Kisch initially found that atrial, but not ventricular, cells contained highly developed Golgi networks, similar to those observed in secretory cells (Kisch 1956). Jamieson and Palade reported that atrial, but not ventricular, myocytes contain spherical, electron opaque granules (Jamieson and Palade 1964). At the same time, physiological experiments conducted by Henry and colleagues revealed that balloon distension of the atria correlated with increased urination in dogs (Henry et al. 1956). In the late 1960s, de Bold and colleagues began characterizing the atrial granules. They found that the content of the granules changed in response to alterations in electrolyte and water balance (de Bold et al. 1981). In a seminal study published in 1981, de Bold and colleagues elegantly linked the seemingly disparate studies of Kisch and Henry by showing that atrial, but not ventricular, extracts contain a potent blood pressure decreasing component that works by stimulating renal sodium and water secretion (de Bold et al. 1981). Thus, the first natriuretic peptide was discovered.

Shortly after the publication of this landmark paper, a number of groups reported the purification and sequencing of atrial peptides of varying sizes that possessed natriuretic, diuretic, and/or smooth muscle relaxing activity (Currie et al. 1984; Flynn et al. 1983; Kangawa et al. 1984; Misono et al. 1984). Several different names were given to these peptides such as atrial natriuretic factor, atriopeptin, cardionatrin, and cardiodilatin. However, atrial natriuretic peptide (ANP) is most often used to describe this peptide in the current literature. The second member of the family to be discovered, B-type natriuretic peptide (BNP), was originally called brain natriuretic peptide because it was purified and sequenced from porcine brain (Sudoh et al. 1988). However, subsequent studies found that it is more highly concentrated in cardiac ventricles of patients with heart failure (Mukoyama et al. 1991; Mukoyama

et al. 1990). Therefore, it is often described as B-type natriuretic peptide today. Finally, the third member of the family, C-type natriuretic peptide (CNP) (Sudoh et al. 1990) was purified in 1991 from porcine brain extracts based on its ability to relax smooth muscle. All three members are similar in primary amino acid structure, contain a 17-residue disulfide ring, and are the products of separate genes.

2 Natriuretic Peptides

2.1 Atrial Natriuretic Peptide

All natriuretic peptides are synthesized as preprohormones (Fig. 1). The human gene encoding ANP is called *NPPA* (GeneID 4878) and is located on chromosome 1 at location 1p36.21. *NPPA* is approximately 2 Kb in length and consists of 3 exons and 2 introns. The resulting mRNA gives rise to a 151 amino acid polypeptide, known as preproANP. The first 25 amino acids constitute a signal sequence that is cleaved to yield a 126 amino acid peptide called proANP, which is the major form of ANP stored in the atrial granules (Oikawa et al. 1984). Upon release from these granules, proANP is rapidly cleaved by corin, a transmembrane cardiac serine protease. Corin is highly expressed on the extracellular surface of atrial cardiomyocytes and has been shown to cleave proANP into the biologically active 28-amino acid form of ANP in vitro (Yan et al. 2000). Mice lacking functional corin have dramatically reduced levels of fully processed ANP in their hearts and are mildly hypertensive (Chan et al. 2005). Alternative processing of proANP in the kidney by an unknown protease results in a 32-amino acid peptide called urodilatin that contains four additional amino-terminal residues (Forssmann et al. 1998). Disruption of the murine ANP gene, *Nppa*, results in marked hypertension, which was initially described as salt-sensitive (John et al. 1995), but later found not to be correlated with dietary salt intake (John et al. 1996).

ANP is well conserved between species (Fig. 2). The 28-amino acid mature form of ANP in humans and rats differs by only one amino acid at position 12, where the human peptide contains a methionine and the rat peptide contains an isoleucine. The mature, circulating form of ANP is identical in humans, chimps, dogs, pigs, horses, and sheep. The sequence of mature rat ANP is identical in mice and rabbits. Additionally, the entire length of the preproANP polypeptide, not just the carboxyl terminal biologically active end, is well conserved. The preproANP polypeptide is similar in many mammalian species, with 100, 86, 81, and 81% homology to the human form in chimpanzees, dogs, mice, and rats, respectively [NCBI Homologene Database].

Several polymorphisms within the *NPPA* coding region have been identified. The T2238C SNP [NCBI SNP Database #rs5065] falls within the coding region and results in a missense mutation that changes the stop codon of preproANP to an arginine, resulting in a preproANP that has two extra arginine residues on the C-terminal end. This same double-arginine pattern is present in the wild-type murine

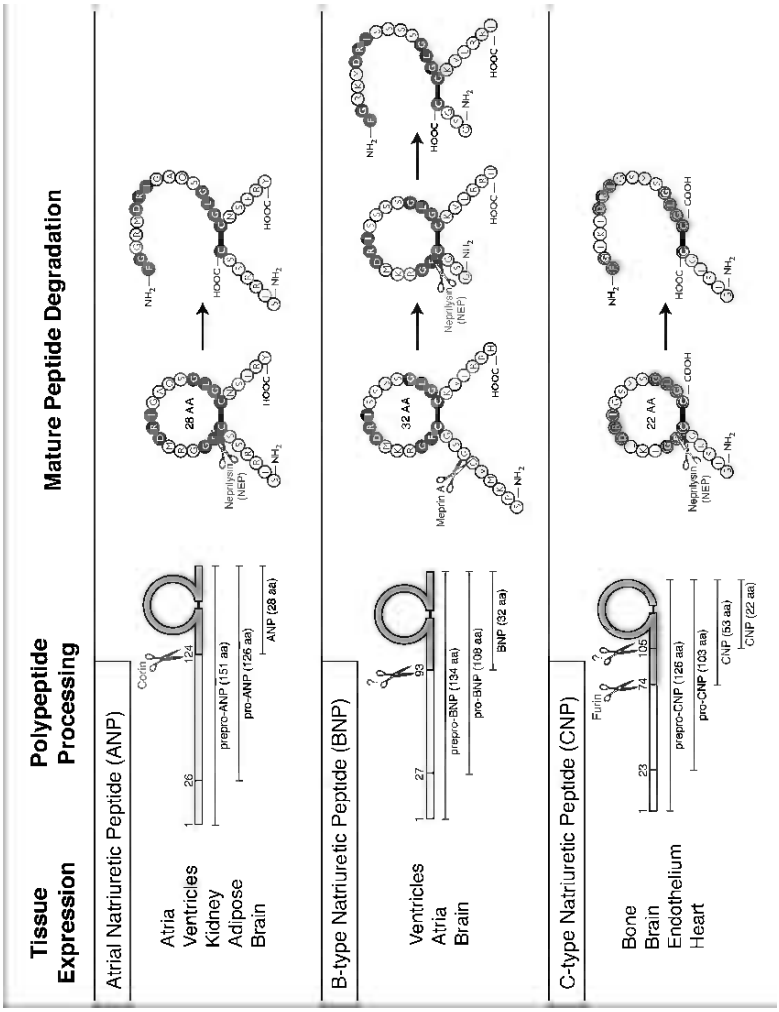

Fig. 1 Structure of the human natriuretic peptides. The structure of the preprohormones for ANP, BNP and CNP are outlined on the left of each panel. The final amino acid sequence and structure of the mature peptides along with the major degradation product are shown on the right. The sites of cleavage are indicated with scissors.

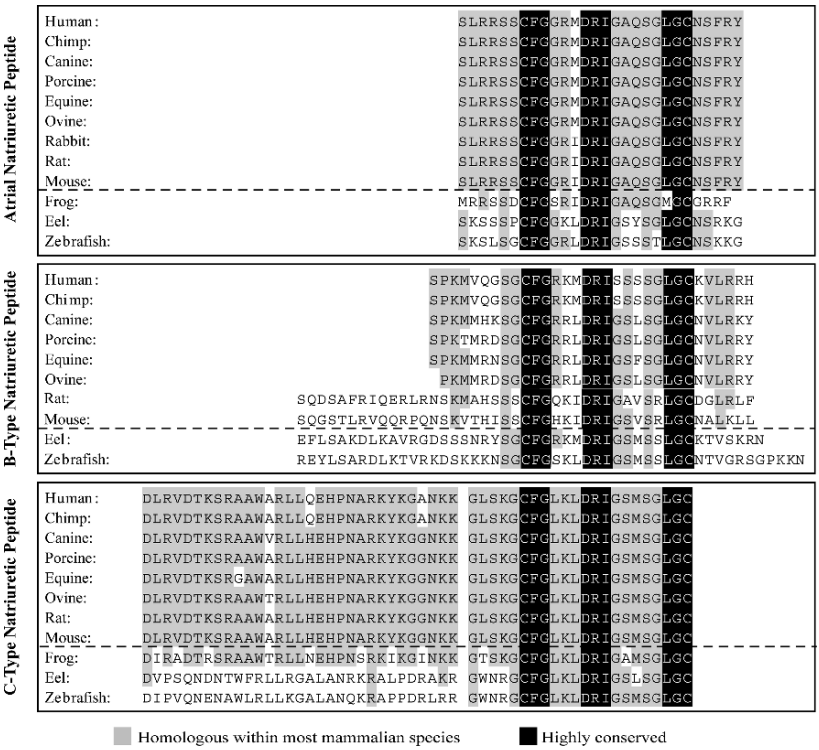

Fig. 2 Sequence alignment of natriuretic peptides. The sequences for the species listed above were obtained from the NCBI Entrez Protein database. The accession numbers for the above sequences (from top to bottom) are: for ANP [NP_006163.1; XP_001141705.1; XP_850357.1; NP_999425.1; NP_999425.1; NP_001075970.1; AAB92564; NP_001075731.1; NP_036744.1; NP_03275.1; P18909; BAA34122; NP_942095], for BNP [NP_002512.1; XP_525186.2; XP_544566.2; P07634; ABG91577; AAB92565; NP_113733.1; NP_032752.1; BAE19674; XP_696498], and for CNP [NP_077720.1; XP_001141992.1; XP_852684.1; NP_001008482.1; XP_001498652; NP_001009479; NP_446202.1; NP_035063.1; BAA04236; BAA13529; XP_692388]. For ANP, the mature circulating form for all mammalian species is 28 amino acid as shown; the same size peptide is shown for the non-mammalian species for comparison purposes only. For BNP, the mature circulating form varies as shown. For CNP, the 53-amino acid form is shown for all.

and rat preproANP, but the mature circulating peptide lacks these two residues. It is not known whether individuals with this polymorphism have circulating ANP that is 30-amino acids in length or if these two arginine residues are cleaved during processing as seen in mice and rats. Three other SNPs cause missense mutations in the same region of preproANP (rs5063, rs13305987, and rs3170926), resulting in V32M, A70V, and L77F substitutions, respectively. This section of proANP is cleaved upon atrial release, and thus the mature circulating form of ANP is not affected. One relatively rare SNP in the Entrez SNP database (rs1803268) causes an arginine to glutamic acid substitution at position 126 of preproANP, which results in a sequence change at the fourth position of the mature 28 residue peptide. The effect of this mutation on ANP function has not been described.

Release of proANP from the atrial granules is primarily stimulated by stretch of the atrial wall caused by increased intravascular volume (Bilder et al. 1986; Edwards et al. 1988; Lang et al. 1985), but pressor hormones also stimulate ANP release (Ruskoaho 2003). Upon secretion and cleavage into the mature peptide, ANP enters the coronary sinus and is distributed to its target organs via the circulation. Plasma levels of ANP are relatively low $(10\,\text{fmol}\,\text{ml}^{-1})$, but in patients with congestive heart failure, circulating ANP levels are elevated from 10- to 30-fold (Burnett et al. 1986; Cody et al. 1986).

The plasma half-life of ANP in humans is approximately 2 min, with reported values falling between 1.7 and 3.1 min (Nakao et al. 1986; Yandle et al. 1986). Degradation of the active ANP peptide occurs through the actions of neutral endopeptidase (NEP) (Stephenson and Kenny 1987; Vanneste et al. 1988) as well as through binding to the natriuretic peptide clearance receptor (NPR-C). NPR-C is a cell surface receptor that lacks guanylyl cyclase activity and controls the local concentrations of natriuretic peptides via constitutive receptor mediated internalization and degradation (see below). Inhibiting NEP, increases the half-life of ANP both in vitro (Stephenson and Kenny 1987; Yandle et al. 1989) and in vivo (Yandle et al. 1989), suggesting that NEP activity contributes to the rapid clearance of ANP. However, it is important to note that mice lacking functional NEP do not exhibit increased natriuretic peptide function (Lu et al. 1995). In contrast, mice lacking NPR-C are hypotensive, exhibit skeletal overgrowth and have reduced ability to clear ANP compared to wild type mice, suggesting that NPR-C is also a physiologic regulator of circulating natriuretic peptide concentrations (Matsukawa et al. 1999).

2.2 B-Type Natriuretic Peptide

BNP was initially purified and sequenced from extracts of porcine brain tissue and hence it was named "brain natriuretic peptide" (Sudoh et al. 1988). Subsequently, BNP was found at much higher concentrations in cardiac tissues (Mukoyama et al. 1991; Mukoyama et al. 1990). The gene encoding human BNP, *NPPB* (GeneID 4879), is located on chromosome 1 at 1p36.2. In the mouse genome, *Nppb* is located on chromosome 4 (Ogawa et al. 1994a). Like *NPPA*, the *NPPB* gene consists of 3 exons and 2 introns (Ogawa et al. 1994a). PreproBNP is 134 amino acids in length, consisting of a 26 amino acid signal sequence followed by 108 amino acids that constitute proBNP. Unlike preproANP, which has high species homology throughout the entire polypeptide sequence, preproBNP sequences in mammals only have high homology at the amino and carboxyl terminal ends of the polypeptide. For example, the homology of canine preproBNP to human preproBNP is 53%, whereas the homology for preproANP between these species is 85%. This lower level of homology gives rise to differing lengths of the active, circulating BNP between mammalian species. For example, in humans and pigs circulating BNP is 32 amino acids in length, while in rats and mice the circulating form is 45 amino acids. The peptidase that cleaves proBNP to its active form has not been identified, but corin is a reasonable suspect.

There are several polymorphisms found in the coding region of human *NPPB* reported in the SNP database. Two, rs35690395 and rs35628673, result in synonymous amino acid residues. Four other SNPs result in changes in the sequence of preproBNP. One (rs5227) results in an R25L substitution of one amino acid before the signal cleavage site. Another mutation (rs5229) results in an R to H substitution at position 47 in the preprohormone sequence, which is the 21st amino acid of proBNP. Mutation rs5230 changes an M to an L at position 93 in preproBNP, corresponding to the 67th amino acid in proBNP. Mutation rs35640285 results in a V to F change at position 94, which is the 68th amino acid of proBNP. None of the above SNPs create changes in the sequence of the mature circulating 32 amino acid form of BNP.

Although low levels of BNP are stored with ANP in atrial granules, BNP is found at greater concentrations in cardiac ventricles. In this tissue, BNP is not stored in granules, but rather transcribed as needed in response to cardiac stress states such as volume overload. The transcription of BNP is under the regulatory control of GATA4, a transcription factor (Grepin et al. 1994; Thuerauf et al. 1994). In normal human subjects, plasma concentrations of BNP are very low (1 fmol ml-1), but in response to congestive heart failure, circulating concentrations of BNP are dramatically elevated (Mukoyama et al. 1991; Mukoyama et al. 1990).

Compared to ANP, circulating BNP has a significantly longer half-life of around 20 min in humans (Mukoyama et al. 1991; Mukoyama et al. 1990). Unlike ANP, BNP is not initially cleaved by NEP. Instead, the first six amino-terminal amino acids of BNP are first cleaved by the metalloprotease, meprin A in the kidney brush border, which then allows further degradation by NEP (Pankow et al. 2007). Like all natriuretic peptides, BNP is also cleared from the circulation by NPR-C.

Both knockout and overexpression models of *Nppb* have been generated in mice. The knockout model of *Nppb* was created by targeted deletion of exons 1 and 2 (Tamura et al. 2000). In contrast to ANP knockout mice, *Nppb*$^{-/-}$ mice showed no signs of systemic hypertension or ventricular hypertrophy on standard or high salt diets. However, *Nppb*$^{-/-}$ mice had ventricular fibrotic lesions that increased in size and number in response to pressure overload, compared to wild type animals. Thus, these studies suggest that BNP is not a regulator of blood pressure, at least in mice. Rather, it is a paracrine regulator of cardiac remodeling. In murine overexpression models of BNP, blood pressure reduction of 20 mmHg was seen with 10- to 100-fold increases in plasma BNP levels (Ogawa et al. 1994a). Interestingly, these mice had marked increases in long bone length compared to their wild-type littermates, which most likely resulted from overactivation of NPR-B, the receptor of CNP (see Sect. 2.3).

2.3 C-Type Natriuretic Peptide

C-type natriuretic peptide (CNP) was initially purified and sequenced from porcine brain extracts (Sudoh et al. 1990). It is the most highly expressed natriuretic peptide

in the brain but is also highly expressed in chondrocytes and endothelial cells. Unlike ANP and BNP, the human gene encoding CNP, *NPPC* (GeneID 4880), is not located on chromosome 1 but on chromosome 2 at location 2q24-qter (Ogawa et al. 1994b). Another difference of NPPC is that it consists of only 2 exons and 1 intron. In the murine genome, *Nppc* is also separated spatially from both ANP and BNP being located on chromosome 2 (Ogawa et al. 1994b). NPPC encodes a polypeptide of 126 amino acids, with a 23 amino acid signal sequence followed by a 103 amino acid proCNP (Tawaragi et al. 1991). PreproCNP shows remarkable homology between species, even more so than preproANP. The preproCNP polypeptides of mammalian species show 99, 96, 91, and 94% homology to the human form in chimpanzees, dogs, mice, and rats, respectively. Perhaps even more telling is that the circulating 22 amino acid carboxyl terminal form of CNP is absolutely identical in all of the above species.

Processing of proCNP to its mature form may occur through the action of the intracellular serine endoprotease, furin. In vitro, furin cleaves the 103 amino acid proCNP into a 53 amino acid carboxyl-terminal biologically active peptide (Wu et al. 2003). This 53 amino acid form of CNP (CNP-53) is the major active form of CNP, at the tissue level (Brown et al. 1997). However, in the systemic circulation, a shorter 22 amino acid form dominates (CNP-22). The protease responsible for this cleavage is not known. Importantly, CNP-53 and CNP-22 appear to bind and activate their cognate receptor, NPR-B, equally well (Yeung et al. 1996).

Within the coding region of NPPC, 4 polymorphisms are reported in the NCBI database, one of which (rs5266) is a synonymous mutation. The G58R SNP (rs13305993) falls in a region of preproCNP that is cleaved during maturation of the polypeptide. The R82Q and A83E polymorphisms (rs5267 and rs13305994) are on the amino terminal side of CNP-53 and would be cleaved upon conversion to CNP-22.

CNP is not stored in granules and its secretion is increased by growth factors (Suga et al. 1993; Suga et al. 1992b) and sheer stress (Chun et al. 1997) in cultured endothelial cells. CNP expression in neointimal vascular smooth muscle cells is increased in response to vascular injury (Brown et al. 1997). In normal human subjects, mean CNP concentration is very low (1 fmol/ml). It is elevated in patients with congestive heart failure, although to a much lower extent than ANP and BNP (Charles et al. 2006; Del Ry et al. 2005; Kalra et al. 2003).

The clearance of CNP-22 in human plasma is very rapid, with a calculated half-life of 2.6 min (Hunt et al. 1994). Like ANP, CNP has been shown in vitro to be inactivated by neutral endopeptidase (Kenny et al. 1993) and is internalized and degraded by NPR-C. The NPR-C route of CNP degradation is especially important at the tissue level, as can be seen in NPR-C knockout models. Mice lacking functional NPR-C receptors have disproportionately long bones, most likely due to the failed clearance of CNP from specific regions in the growth plate (Jaubert et al. 1999; Matsukawa et al. 1999).

Both over and under expression models of CNP have been created in mice. Knockout animals were generated by disruption of exons 1 and 2 of *Nppc* (Chusho et al. 2001). The $Nppc^{-/-}$ mice are dwarfs due to impaired endochondral

ossification and have severely reduced life spans. Targeted expression of CNP in the growth plate chondrocytes rescued the skeletal defect of *Nppc*$^{-/-}$ mice and prolonged their survival. Importantly, the *Nppc* transgene partially rescued skeletal growth in a murine model of achondroplasia caused by a gain of function mutation in the fibroblast growth factor receptor 3 ($Fgfr3^{\mathrm{ach}}$), which accounts for most types of human dwarfism and may indicate that CNP analogs could be used to treat various forms of human dwarfism (Yasoda et al. 2004). A recent spontaneous point mutation in *Nppc* was characterized by a dwarfism phenotype similar to that seen in the *Nppc*$^{-/-}$ mice (Jiao et al. 2007). The mutation resulted in a missense mutation (R117G) in proCNP, which is one of the highly conserved residues in the cysteine ring found in all natriuretic peptides. Our recent data indicate that the mutant CNP has reduced ability to bind and activate NPR-B (Yoder, 2008). Finally, balanced translocations of chromosome 2, which separate putative negative transcriptional regulators from the CNP promoter, cause abnormal elevations in CNP and associated Marfornoid-like skeletal overgrowth in humans (Bocciardi et al. 2007; Moncla et al. 2007). Hence, moderate elevations of CNP are correlated with demonstrative skeletal overgrowth.

3 Natriuretic Peptide Receptors

There are three known natriuretic peptide binding proteins. All members contain a relatively large ($\sim$450 amino acid) extracellular ligand binding domain and a single membrane-spanning region of about 20 residues. Natriuretic peptide receptors A and B contain an equally large intracellular domain consisting of a so-called kinase homology domain, dimerization domain, and carboxyl-terminal guanylyl cyclase domain. Thus, NPR-A and NPR-B signal by catalyzing the synthesis of the intracellular signaling molecule cGMP. In contrast, NPR-C only contains a 37 residue intracellular domain and lacks guanylyl cyclase activity. It primarily controls local natriuretic peptide concentrations via receptor-mediated internalization and degradation, although many groups have reported signaling functions for NPR-C as well (Rose and Giles 2008).

3.1 Natriuretic Peptide Receptor-A

Natriuretic peptide receptor-A (NPR-A) is the principal receptor of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP). Its extracellular domain contains three intramolecular disulfide bonds and five N-linked glycosylation sites (Miyagi and Misono 2000; Miyagi et al. 2000). NPR-A exists as a homodimer or homotetramer in its native state, and oligomerization is ligand-independent (Chinkers and Wilson 1992; Iwata et al. 1991), although ligand binding does bring the juxtamembrane regions of each monomer closer together (Labrecque et al. 2001). NPR-A binds natriuretic peptides at a stoichiometry of 2:1 with a rank natriuretic peptide

preference of: ANP ⩾ BNP ≫ CNP (Bennett et al. 1991; Koller et al. 1991; Suga et al. 1992a). The human NPR-A gene is located on chromosome 1q21–22 and consists of 22 exons and 21 introns within 16 kilobases (Lowe et al. 1990; Takahashi et al. 1998). The murine NPR-A gene, *Npr1*, is located on chromosome 3.

Under basal conditions, NPR-A is phosphorylated on four serine and two threonine residues located in the N-terminal portion of the kinase homology domain (Potter and Hunter 1998b). Phosphorylation is essential for activation of NPR-A and dephosphorylation is a mechanism of desensitization in response to prolonged ANP exposure or protein kinase C activation (Potter and Garbers 1992, 1994). Although ATP increases ANP-dependent guanylyl cyclase activity, the mechanism for this effect is debatable (Antos et al. 2005; Antos and Potter 2007; Burczynska et al. 2007; Joubert et al. 2005). Our recent data indicate that ATP reduces the K_m for NPR-A (Antos and Potter 2007). NPR-A internalization and degradation is also controversial. One group consistently reports that the majority of internalized ANP-NPR-A complexes are degraded via a lysosomal pathway with a small portion returning intact to the plasma membrane (Pandey 2002). Meanwhile, studies in primary kidney, Chinese Hamster ovary, and HEK293 cells indicate that NPR-A is a membrane resident protein that does not undergo acute internalization and degradation (Fan et al. 2005; Koh et al. 1992; Vieira et al. 2001).

NPR-A and/or its mRNA is expressed in kidney, lung, adipose, adrenal, brain, heart, testis, and vascular smooth muscle tissue (Goy et al. 2001; Lowe et al. 1989; Nagase et al. 1997; Wilcox et al. 1991). NPR-A null mice exhibit chronic salt-resistant hypertension and cardiac hypertrophy and fibrosis (Kuhn et al. 2002; Lopez et al. 1995; Oliver et al. 1997). A deletion in the human NPR-A gene was identified in nine Japanese individuals, of which eight had essential hypertension; the normotensive individual with the altered allele had left ventricular hypertrophy (Nakayama et al. 2000). To our knowledge, this study has not been repeated.

3.2 Natriuretic Peptide Receptor-B

Natriuretic peptide receptor-B (NPR-B) is the principal receptor of C-type natriuretic peptide (CNP) and exhibits similar topology, glycosylation, and intramolecular disulfide bonding patterns as NPR-A. The extracellular and intracellular regions of rat NPR-B are 43 and 78% identical to rat NPR-A at the amino acid level, respectively (Schulz et al. 1989). NPR-B binds natriuretic peptides with a selectivity preference of CNP ≫ ANP ⩾ BNP (Bennett et al. 1991; Koller et al. 1991; Suga et al. 1992a). The human NPR-B gene is located on chromosome 9p12–21 and the murine version, *Npr2*, is located on chromosome 4 (Lowe et al. 1990).

Four serine and one threonine phosphorylation sites have been identified within the amino-terminal portion of NPR-B's kinase homology domain (Potter and Hunter 1998a), and receptor dephosphorylation has been shown to mediate desensitization in response to prolonged CNP exposure, protein kinase C activation, and

intracellular calcium elevations (Potter 1998; Potter and Hunter 2000; Potthast et al. 2004). ATP increases the guanylyl cyclase activity of NPR-B, by decreasing its Michaelis constant (Antos and Potter 2007).

NPR-B and/or its mRNA is expressed in bone, brain, fibroblasts, heart, kidney, liver, lung, uterine, and vascular smooth muscle tissue (Bryan et al. 2006; Chrisman et al. 1993; Dickey et al. 2007; Herman et al. 1996; Langub et al. 1995). Mice with a targeted disruption of the NPR-B gene, display dwarfism and female sterility (Tamura et al. 2004). The achondroplastic (cn/cn) mouse has a spontaneous mutation in NPR-B resulting in the substitution of a highly conserved Leu with Arg in the guanylyl cyclase domain, which inactivates the enzyme. Endochondral ossification is disrupted in mice with two defective alleles, leading to dwarfism (Tsuji and Kunieda 2005). However, these mice exhibit no fertility defect. NPR-B dominant negative mutant transgenic rats have also been generated (Langenickel et al. 2006). In addition to mild growth retardation of the long bones, the rats displayed progressive, blood pressure-independent cardiac hypertrophy and an elevated heart rate. Consistent with a prominent role for CNP in the heart, NPR-B, not NPR-A, is the most active natriuretic peptide receptor in the failed heart (Dickey et al. 2007). Homologous loss-of-function mutations in human NPR-B result in a rare form of dwarfism called acromesomelic dysplasia, type Maroteaux (AMDM) (Bartels et al. 2004). Individuals with single defect NPR-B alleles are statistically shorter than the average person (Olney et al. 2006).

3.3 Natriuretic Peptide Receptor-C

Natriuretic peptide receptor-C (NPR-C) consists of a large extracellular ligand-binding domain that is approximately 30–35% identical to NPR-A and NPR-B, a single membrane-spanning region, but only 37 intracellular amino acids (Chang et al. 1989; Fuller et al. 1988; Porter et al. 1990). Unlike NPR-A and NPR-B, it contains one or two juxtamembrane intermolecular disulfide bonds. Hence, it is a disulfide-linked dimer. It has no known enzymatic activity but has been suggested to signal in a G protein-dependent manner (Rose and Giles 2008). It contains three known N-linked extracellular glycosylation sites (Stults et al. 1994) and binds natriuretic peptides with a stoichiometry of two molecules of receptor to one molecule of ligand (Ammarguellat et al. 2001). Its ligand selectivity preference is: ANP $>$ CNP $\geqslant$ BNP (Bennett et al. 1991; Suga et al. 1992a). Compared to NPR-A and NPR-B, NPR-C has much less stringent specificity for structural variants of ANP and will bind with high affinity to ring-deleted ANP analogs (Maack et al. 1987). The main function of NPR-C, also known as the clearance receptor, is to clear circulating natriuretic peptides through the process of receptor-mediated internalization and degradation (Koh et al. 1992; Nussenzveig et al. 1990). Internalization of NPR-C occurs in the absence of ligand; thus, this is a constitutive process (Nussenzveig et al. 1990). Osteocrin, an endogenous protein with limited homology to members of the natriuretic peptide family, binds NPR-C, but not NPR-A or

NPR-B (Moffatt et al. 2007). Osteocrin is thought to compete with CNP for binding to NPR-C in bone, and therefore, increase local CNP levels during critical periods for bone development (Moffatt et al. 2007). The human NPR-C gene is located on chromosome 5p13–14 and contains 8 exons and 7 introns spanning more than 65 kilobases (Lowe et al. 1990; Rahmutula et al. 2002). *Npr3*, the murine version of the NPR-C gene, is located on chromosome 15. A splice variant of NPR-C containing an additional cysteine residue has also been identified and characterized from bovine lung (Mizuno et al. 1993).

NPR-C is the most widely and abundantly expressed natriuretic peptide receptor; for example, it constitutes ~94% of the total ANP binding sites in endothelial cells (Leitman et al. 1986). NPR-C and/or its mRNA is expressed in adrenal, brain, heart, kidney, mesentery, and vascular smooth muscle tissue (Nagase et al. 1997; Porter et al. 1990; Suga et al. 1992c; Wilcox et al. 1991). NPR-C knockout mice exhibit increased ANP half-lives, long bone overgrowth, hypotension, mild diuresis, dilute urine, and blood volume depletion (Matsukawa et al. 1999). Mouse strains containing chemically induced loss-of-function mutations in the extracellular domain of NPR-C display skeletal overgrowth from endochondral ossification defects as well (Jaubert et al. 1999).

4 Physiologic Effects of Natriuretic Peptides

Natriuretic peptides and their receptors mediate a diverse array of physiologic effects ranging from blood pressure control to endochondral ossification. This broad assortment of responses is achieved from the distinct actions of individual natriuretic peptides interacting with specific guanylyl cyclase receptors. This is particularly apparent in mice with targeted deletions of individual natriuretic peptides or natriuretic peptide receptors. The following section will highlight work describing the effects of natriuretic peptides on the cardiovascular system and bone growth.

4.1 Natriuretic Peptide Effects on Blood Pressure

ANP binding to NPR-A is a key-signaling pathway, which regulates normal homeostatic blood pressure. This is clearly demonstrated in mice lacking ANP or its receptor NPR-A, which have blood pressures that are elevated 20–40 mm mercury, compared to control mice (John et al. 1995; John et al. 1996; Lopez et al. 1995; Oliver et al. 1997). The link between NPR-A and blood pressure in mice is particularly strong because Smithies and colleagues demonstrated that NPR-A copy number is inversely related to blood pressure in a remarkably linear manner (Oliver et al. 1998). Conversely, blood pressures in transgenic mice overexpressing ANP or BNP are substantially decreased (Ogawa et al. 1994a; Steinhelper et al. 1990). Although infusion of supraphysiological levels of CNP into animals acutely decreases blood pressure (Clavell et al. 1993; Sudoh et al. 1990), mice lacking functional

CNP or NPR-B are normotensive (Chusho et al. 2001; Tamura et al. 2004), suggesting that the CNP/NPR-B pathway is not a fundamental regulator of basal blood pressure in mice.

NPR-A dependent decreases in blood pressure are achieved through natriuresis and diuresis, vasorelaxation, increased endothelium permeability, and antagonism of the renin-angiotensin system. Classic experiments showed that atrial extract infusions resulted in rapid renal excretion of water and sodium (de Bold et al. 1981). Studies by Garbers and colleagues indicated that the renal response requires NPR-A because mice lacking this receptor do not respond to ANP, BNP, or to acute volume expansion (Kishimoto et al. 1996). Similar studies found that NPR-A was also required for ANP- or BNP-dependent vasorelaxation in mice (Lopez et al. 1997). Physiological experiments involving mice with severe reductions of NPR-A in vascular smooth muscle cells demonstrated that while smooth muscle NPR-A is required for acute ANP- or BNP-dependent vasorelaxation, this response does not play a significant role in controlling chronic blood pressure (Holtwick et al. 2002).

The ability of the ANP/NPR-A pathway to increase endothelial permeability is supported by the observation that hematocrit levels are elevated prior to urination and are preserved in nephrectomized animals (Almeida et al. 1986; Fluckiger et al. 1986; Richards et al. 1988). Furthermore, mice with genetically engineered reductions of NPR-A in vascular endothelium exhibit volume expansion, hypertension, and reduced albumin clearance from the vascular system (Sabrane et al. 2005).

4.2 Effects of Natriuretic Peptides on Cardiac Hypertrophy and Fibrosis

In addition to regulating blood pressure, natriuretic peptides inhibit cardiac hypertrophy and remodeling. Hypertrophy is regulated by ANP and NPR-A, whereas remodeling is regulated by both the ANP/BNP/NPR-A and the CNP/NPR-B pathways.

Although prolonged hypertension can cause hypertrophy, the level of hypertrophy in NPR-A deficient mice is significantly greater than that observed in other genetic models that cause similar levels of hypertension, suggesting that NPR-A elicits a local growth inhibitory signal in the heart. Data for this idea was initially shown in NPR-A knockout mice, which have enlarged hearts even when effectively treated with antihypertensive drugs from birth (Knowles et al. 2001). Additional studies determined that transgenic reexpression of NPR-A in the hearts of NPR-A$^{-/-}$ mice reduced cardiomyocyte size without affecting heart rate or blood pressure (Kishimoto et al. 2001). Finally, mice with reduced cardiomyocyte expression of NPR-A exhibited moderate hypertrophy even though they were slightly hypotensive (Holtwick et al. 2003; Patel et al. 2005). In terms of natriuretic peptides, mice lacking ANP have larger hearts, whereas mice transgenically overexpressing ANP have smaller hearts (Barbee et al. 1994; Steinhelper et al. 1990). In contrast, targeted deletion

of BNP resulted in normotensive mice with normal heart size but with increased ventricular fibrosis – especially when subjected to pressure overload (Tamura et al. 2000). Thus, genetic studies in mice strongly support a role for ANP activation of NPR-A in the local inhibition of cardiac hypertrophy and BNP activation of NPR-A in the inhibition of cardiac fibrosis.

Recently, data supporting a role for the CNP/NPR-B pathway in cardiac remodeling has been reported. Although NPR-B inactivation mutations in mice have not been shown to cause hypertrophy (Tamura et al. 2004; Tsuji and Kunieda 2005), transgenic rats expressing a dominant negative form of NPR-B exhibit mild blood pressure-independent cardiac hypertrophy and increased heart rate (Langenickel et al. 2006). In addition, CNP infusion was shown to reduce cardiac remodeling in response to experimentally induced myocardial infarction in rats, and transgenic expression of CNP improved outcomes in mice subjected to ischemia/reperfusion injury or myocardial infarction (Wang et al. 2007).

4.3 Effects of CNP and NPR-B on Bone Growth

The most obvious function of the CNP/NPR-B pathway is to stimulate long bone growth. Though undetectable at birth, mice lacking functional CNP or NPR-B develop dwarfism due to impaired endochondrial ossification (Chusho et al. 2001; Tamura et al. 2001; Tsuji and Kunieda 2005). Conversely, transgenic CNP overexpression or reduced degradation of CNP due to loss of function mutations in NPR-C result in skeletal overgrowth (Jaubert et al. 1999; Matsukawa et al. 1999; Yasoda et al. 2004). Growth plate histology reveals that the endochondral proliferative and hypertrophic zones are reduced in mice with impaired CNP or NPR-B signaling, whereas overexpressing mice have enlarged growth plates (Chusho et al. 2001; Tamura et al. 2004; Yasoda et al. 2004). One cGMP effector involved in the long bone growth pathway is cGMP-dependent protein kinase II, also known as PKGII or cGKII. Loss of function mutations in the mouse or rat gene that encodes this kinase also cause dwarfism (Chikuda et al. 2004; Pfeifer et al. 1996). Interestingly, the growth plates of rodents with defective cGKII are enlarged, which differs from the diminished growth plates seen in the CNP or NPR-B deleted mice, suggesting that a cGKII-independent pathway is also involved in CNP-dependent long bone growth.

Humans with two loss of function alleles for NPR-B suffer from a rare type of autosomal recessive dwarfism, called acromesomelic dysplasia, type Maroteaux (Bartels et al. 2004). These individuals are characterized by disproportionate limb to torso ratios that are only obvious a year or more after birth. Interestingly, although single copy carriers of a nonfunctional NPR-B allele do not suffer from disease, they are statistically shorter than comparable individuals with two wild type NPR-B alleles (Olney et al. 2006). Thus, it is possible that NPR-B mutations could have a significant effect on the stature of the general population.

5 Therapeutics of Natriuretic Peptides

The Encyclopedia Britannica defines therapeutics as "the treatment and care of a patient for the purpose of both preventing and combating disease". By these criteria, natriuretic peptides have already found their way into the clinical arsenal – especially ANP and BNP. Measurement of serum BNP levels is used in the clinic as a diagnostic indicator for heart failure, and synthetic forms of both of these peptides have been approved in some countries for the treatment of heart failure (Gardner 2003). The extent of their usefulness, however, has come under question due to their limited renal actions, and trials are underway to determine the most effective use of these peptides. In this section, we will explore the history of both synthetic ANP and BNP as therapeutic agents.

5.1 Synthetic ANP (Anaritide and Carperitide)

As previously discussed, the natriuretic peptide field emerged with the key discovery that specific peptides present in atrial extracts cause natriuresis and diuresis. Initially, numerous peptide variants were identified in rat atrial extracts, but subsequent studies revealed that the mature form of ANP is 28-amino acids and that smaller versions are degradation products that maintain various levels of activity. The most widely studied of these is the 25-amino acid peptide lacking the first three amino-terminal residues. This peptide is referred to as ANF IV and its synthetic form is called anaritide.

Since the activities of the 25-amino acid and mature 28-amino acid peptide were similar, many studies were conducted with the smaller peptide. Studies by Cody and colleagues indicated that infusion of anaritide in healthy male volunteers resulted in natriuresis, diuresis, and reduction in systolic blood pressure; however, in seven patients with congestive heart failure, the changes in urine volume and sodium excretion were minimal (Cody et al. 1986). Saito and colleagues observed a similar lack of diuresis and natriuresis, when congestive heart failed patients were infused with the mature form of ANP (Saito et al. 1987). Meanwhile, others acknowledged the renal hyporesponsiveness to anaritide in congestive heart failed patients, but indicated that the renal parameters did show a statistically significant increase in larger patient samples (Fifer et al. 1990). In Japan, clinical studies on the effectiveness of mature ANP continued; and in 1995, synthetic full length ANP (carperitide) was approved for the treatment of acute decompensated heart failure. In the United States, clinical use of BNP, not ANP, was explored for the treatment of heart failure due to its larger renal responsiveness, and possibly due to unique patient opportunities.

Investigations were also initiated to study the effectiveness of ANP in the treatment of human renal disease. Specifically, trials were conducted to evaluate the ability of anaritide infusion to reduce the need for dialysis in patients with acute tubular necrosis. The initial study with 53 patients suggested a positive outcome for patients receiving anaritide because they had increased creatinine clearance and a decreased

need for dialysis (Rahman et al. 1994). This led to the formation of a multicenter placebo-controlled clinical trial in 504 patients with acute tubular necrosis. While 24-h infusion of anaritide did not improve the overall survival of the patients without dialysis, it appeared that a subset of patients might have benefited (Allgren et al. 1997). Thus, a second trial was conducted in patients with oliguric acute renal failure (renal failure resulting in the production of less than 400 ml of urine per 24 h). However, this 222 patient trial indicated no statistically significant benefit of anaritide in dialysis-free survival (Lewis et al. 2000). Both trials remarked on the severe hypotension that often occurred as a result of the anaritide infusion. In fact, it is this severe hypotension that appears to be limiting the utility of anaritide or nesiritide (see Sect. 5.2) as a therapy for either heart failure or renal disease. The authors stated in their discussion, "it is possible that if this hypotension could have been avoided, anaritide would have been efficacious" (Lewis et al. 2000).

Anaritide was also investigated for its ability to prevent radiocontrast-induced nephropathy. This form of nephropathy is a common cause of acute renal failure in the hospital setting and is defined as acute renal failure – or more specifically, an increase in blood urea nitrogen of 50% or 20 mg/dL or an increase in serum creatinine levels of 1 mg/dL within 24 h or both - occurring within 48 h of exposure to intravascular radiographic material that is not attributable to other causes (Barrett and Parfrey 1994). However, in a 247 person clinical trial anaritide along with hydration was no more effective at preventing radiocontrast-induced nephropathy than hydration alone (Kurnik et al. 1998).

Finally, in 2004, studies conducted in Sweden compared the ability of the loop diuretic, furosemide, or mature ANP (1–28) to increase GFR, renal blood flow, and reduce renal oxygen consumption in patients with acute renal failure. However, they concluded that furosemide was a more effective agent (Sward et al. 2005). Therefore, despite its potent natriuretic and diuretic effects in normal, healthy subjects, clinical studies conducted to date indicate little or no therapeutic benefit of ANP analogs in the successful treatment of renal disease.

5.2 Synthetic BNP (Nesiritide)

Given the natriuretic effects of ANP, the related peptide BNP, was assumed to elicit a similar response. McGregor and colleagues demonstrated that administration of porcine BNP resulted in a natriuretic response and an increase in urinary excretion of cGMP (McGregor et al. 1990). Yoshimura and colleagues reported the same response in healthy volunteers to infusion of human BNP (Yoshimura et al. 1991). Furthermore, patients with congestive heart failure also responded to infusion of BNP.

The effectiveness of 24-h infusion of nesiritide to patients with congestive heart failure was examined in a multicenter, placebo-controlled trial. The peptide resulted in a reduction of both preload and afterload resulting in an increase in stroke volume and cardiac output (Mills et al. 1999). The results of a second multicenter trial, called the Vasodilation in the Management of Acute Congestive Heart Failure

(VMAC) study, compared the effects of the addition of nitroglycerin or nesiritide versus placebo to standard therapy. The group treated with nesiritide had improved dyspnea after 3 h treatment, while there was no difference in the other groups. The nitroglycerin group reported more adverse effects than the nesiritide group. Additionally, patients receiving nesiritide had less adverse cardiovascular effects at either the 0.015 or 0.03 μg/kg/min infusion rate compared to patients receiving dobutamine as determined by the 246-patient PRECEDENT Trial (de Lissovoy et al. 2003).

The results of these studies likely led to the approval of nesiritide, marketed under the trade name Natrecor, for the treatment of acute decompensated heart failure in the United States in 2001. In 2003, nesiritide became commercially available in Israel and Switzerland, under the trade name Noratak. However, approval in the rest of Europe was delayed pending further investigations into the renal responses of nesiritide infusion – a request that in retrospect is prophetic.

With the approval of the first new intravenous compound for the treatment of heart failure in many years, use of nesiritide was immediate. After approval, the number of patients treated with nesiritide was larger than any clinical trial and with the larger sample population came some unpleasant findings. Initially, Wang and colleagues reported in 2004 that nesiritide does not improve renal function in patients with chronic heart failure (Wang et al. 2004), but more damaging were two meta-analysis studies by Sackner-Bernstein and colleagues indicating that nesiritide worsened renal function and increased the likelihood of death (Sackner-Bernstein et al. 2005a, b).

The results of a 75-person study (BNP-CARDS study), however, suggest nesiritide has no detrimental effect on renal function, when cohorts of similar baseline renal function were compared (Witteles et al. 2007). The number of persons in this study was small, however, so a more definitive conclusion on whether nesiritide impairs renal function will have to wait until the result of more detailed, larger studies are released. Several such studies are currently in progress. One is a clinical trial enlisting at least 1,900 patients throughout Europe and Latin America – the ETNA (Evaluating Treatment with Nesiritide in Acute Decompensated Heart Failure) trial. This trial was scheduled to begin in 2006 to study the efficacy of nesiritide on treatment of acutely decompensated heart failure. Results from the trial are not yet available. The second study involving about 900 patients, called FUSION II, was conducted to determine the safety and efficacy of outpatient administration of nesiritide to patients with heart failure. Preliminary analysis indicates that nesiritide did not induce renal complications or increase patient mortality (Cleland et al. 2007). Finally, there is the ASCEND HF trial (Acute Study of Clinical Effectiveness of Nesiritide in Decompensated Heart Failure). This trial is scheduled to compare the effects of nesiritide treatment versus placebo for a minimum of 24 h up to a maximum of 7 days in 7,000 heart failure patients.

Meanwhile, other therapeutic applications of nesiritide have also been investigated. Given that nesiritide was often reported to decrease pulmonary capillary wedge pressure, Michaels and colleagues tested its effectiveness in pulmonary hypertension (Michaels et al. 2005). However, they found no effect of a 30 min infusion. Chen and colleagues have investigated the effectiveness of subcutaneous

injections of nesiritide. Their most recent paper on effects in a dog heart failure pacing model suggest that subcutaneous injection of nesiritide reduces both preload and afterload but has no effect on cardiac output (Chen et al. 2006).

5.3 Clinical Conclusions

Expectations were high when the natriuretic peptides were first approved for the treatment of acute decompensated heart failure. However, their effectiveness is clearly connected to the clinical situations in which they are used. As described above, current trials are underway to more effectively define these settings. Some of the limitations were foretold in previous studies. For example, in 1990 Fried and colleagues concluded that anaritide-induced decreases in mean arterial pressure may "limit its therapeutic potential" (Fried et al. 1990). Concerns were voiced again in 2000, when Lewis and colleagues concluded that anaritide "may be beneficial if the hypotension could be avoided" (Lewis et al. 2000). Some of these limitations of anaritide may also be true of nesiritide, even though it has higher reported renal responses in congestive heart failed patients. Nonetheless, despite current reservations about nesiritide, it is likely that it or new and improved forms will find clinical usefulness once dosage and other clinical parameters regarding efficacy and safety are optimized. For example, CD-NP, a chimeric designer peptide consisting of the carboxyl tail from Dendroaspis natriuretic peptide fused to full length CNP, retains the beneficial renal effects of BNP while being substantially less hypotensive (Lisy et al. 2008). Interestingly, the unique properties of CD-NP result, at least in part, from its ability to activate both NPR-A and NPR-B (Dickey et al, submitted). Hence, CD-NP is the first dual natriuretic peptide receptor activator.

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Cyclic GMP-Hydrolyzing Phosphodiesterases

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1 Introduction

The cyclic nucleotides (cN), cAMP and cGMP, are key second messengers that mediate the intracellular effects of many signals known as "first messengers", including environmental signals such as photons for vision and chemicals for taste, as well

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as hormones, paracrine factors, neurotransmitters, or autocrine factors. Many "first messengers" affect the level of cAMP and/or cGMP thereby initiating increased or decreased signaling selectively through cAMP- or cGMP-signaling pathways. cAMP and cGMP are synthesized by adenylyl cyclases and guanylyl cyclases, respectively, and degraded by a large superfamily of cN phosphodiesterases (PDE). Following the discovery of cAMP (Rall and Sutherland 1958), the search for the role of cAMP in modulating a wide variety of biological processes quickly ensued. Cyclic GMP was discovered in 1963, but many years elapsed before cGMP signaling proved to be a powerful and selective physiological process in its own right. Sutherland and colleagues reasoned that for cNs to be biologically relevant, there must be a mechanism for their removal from the cellular milieu. In 1958 they reported that heart extracts contained PDE activity that dampened or terminated the action of cAMP, and this PDE activity was blocked by methylxanthines, i.e., caffeine and theophylline (Butcher and Sutherland 1962). This was the first report of PDE action and identification of PDE inhibitors.

In mammals, cAMP effects are mediated by a number of intracellular proteins including cAMP-dependent protein kinases (PKA), cAMP-gated cation channels, cAMP-regulated guanine nucleotide-exchange factors (GEF), and the catalytic sites of certain cN PDEs. Intracellular targets for cGMP include cGMP-dependent protein kinases (PKG), cGMP-gated cation channels, allosteric cGMP-binding sites on a subgroup of PDEs (cGMP-binding PDEs), and the catalytic sites of certain PDEs. Many of these proteins (PKA, PKG, cN-gated cation channels, GEFs, and allosteric cGMP-binding sites in certain PDEs), show high specificity for either cAMP or cGMP, although "cross-activation" of a cN-binding protein can also occur in some cases. Catalytic sites of PDEs vary in cN specificity; some are highly specific for either cAMP or cGMP while others are less discriminating between the cNs. Cyclic AMP-signaling pathways play a critical role in either mediating or modulating cellular functions in practically all mammalian tissues. The tissue distribution and action of the cGMP-signaling pathways are apparently much more restricted. Cyclic GMP signaling is particularly prominent in modulating smooth muscle tone and proliferation, inhibition of platelet aggregation, vision, water and electrolyte homeostasis, and bone growth.

The balance between activities of cyclases and PDEs largely determines intracellular cN levels, and the activities of these enzymes are subject to complex and dynamic regulatory processes. The hydrolytic activities of PDEs provide the primary counterpoint to the synthesis of cNs by the cyclase activities and act to dampen or terminate a cN signal. Although cNs can be extruded from the cell by some members of the family of multidrug resistance proteins, evidence suggests that this process is slow and quantitatively minor compared to the catalytic action of PDEs. The activity of a cN-signaling pathway can be altered either by an increase or decrease in the intracellular level of that cN and/or by changes in cN level within spatially distinct sub-compartments within a cell. Most "first messengers" interact with specific receptors on the cell surface. In contrast, nitric oxide (NO), a "first messenger" that activates cGMP-signaling, traverses the plasma membrane to directly increase

the activity of NO-sensitive guanylyl cyclases. The action of "first messengers" can selectively increase activity in either the cyclases or PDEs, but in many instances, the activities of both types of enzymes are altered either directly or indirectly.

1.1 Action and Regulation of Cyclic Nucleotide Phosphodiesterases

1.1.1 Action of the PDE Superfamily

PDEs hydrolyze the cyclic phosphate ring that is unique to cAMP and/or cGMP by insertion of a solvent derived hydroxyl at the phosphorous atom in the 6-member cyclic phosphate ring. The product of this reaction, either 5′-AMP or 5′-GMP, is inactive as a second messenger in cN-signaling pathways. Breakdown of either cN in a cell, in particular cellular compartments, or in response to an extracellular signal can involve PDEs that are highly specific for cAMP or cGMP as well as PDEs that hydrolyze both cAMP and cGMP.

1.1.2 Regulation of PDEs

PDEs are highly regulated by inputs from many pathways. Integration of these signals modulates the catalytic activities of PDEs and in doing so provides for negative feedback control of cN levels in many cells, cross-talk between disparate signaling pathways, and regulation of the intensity and duration of the cellular response to external stimuli. PDEs are regulated by numerous processes including intracellular cAMP and cGMP levels, phosphorylation/dephosphorylation, selective tethering in subcellular compartments, alterations in PDE protein level, and interactions with other proteins such as calmodulin, transducin, anchoring proteins, receptor for activated protein kinase C, β-arrestins, and tyrosine kinases.

1.1.3 Classification, Nomenclature, and Tissue Distribution of PDEs

All mammalian PDEs are Class I PDEs and have been subdivided into 11 families (PDE1-PDE11) (Beavo et al. 2006; Bender and Beavo 2006; Bolger 2006; Conti and Beavo 2007). Human PDEs comprise a complex superfamily of enzymes derived from 21 genes. The subfamilies, or genes, within a family are named using upper case letters, e.g., A, B, and C in PDE1A, PDE1B, and PDE1C; forms produced by RNA splicing are termed variants and named using Arabic numbers, e.g., the term 1 at the end of PDE1A1. Some families (PDE2, PDE5, PDE8, PDE9, PDE10, and PDE11) contain products of a single gene; others (PDE1, PDE3, PDE4, PDE6, and PDE7) contain products of multiple genes. PDEs are most similar in the ∼270 amino acids that comprise the catalytic (C) domains where they share ∼20–50%

amino acid sequence identity; sequence identity among members of a single family is much higher (70–80%). Although members of a PDE family share many similarities, they can differ in relative specificities and affinities for cGMP and cAMP, inhibitor specificities, and regulatory mechanisms; full comparison of functional characteristics among members within the respective PDE family, including potencies of "selective" inhibitors, has not yet been comprehensively conducted. PDEs 4, 7, 8 are highly specific for cAMP, PDEs 5, 6, 9 are highly specific for cGMP, and the remaining five (PDEs 1, 2, 3, 10, and 11) hydrolyze both cNs with varying efficiencies (Fig. 1 and Table 1) (Beavo et al. 2006; Bender and Beavo 2006; Conti and Beavo 2007). Structural and functional characteristics of PDEs that hydrolyze cGMP are depicted in Fig. 1 and Table 1. PDE2 is often referred to as the cGMP-stimulated cAMP PDE, but it hydrolyzes both cNs with approximately equal efficiency. Likewise, PDE3 is often referred to as the cGMP-inhibited PDE, which is the case if cAMP is the substrate. However, cGMP is also a good substrate for PDE3 (Degerman et al. 1996). In the PDE1 family, PDE1A and PDE1B significantly prefer cGMP as substrate (20:1 and 5:1), while PDE1C utilizes cAMP and cGMP with equal affinity and efficiency (Zhao et al. 1997a).

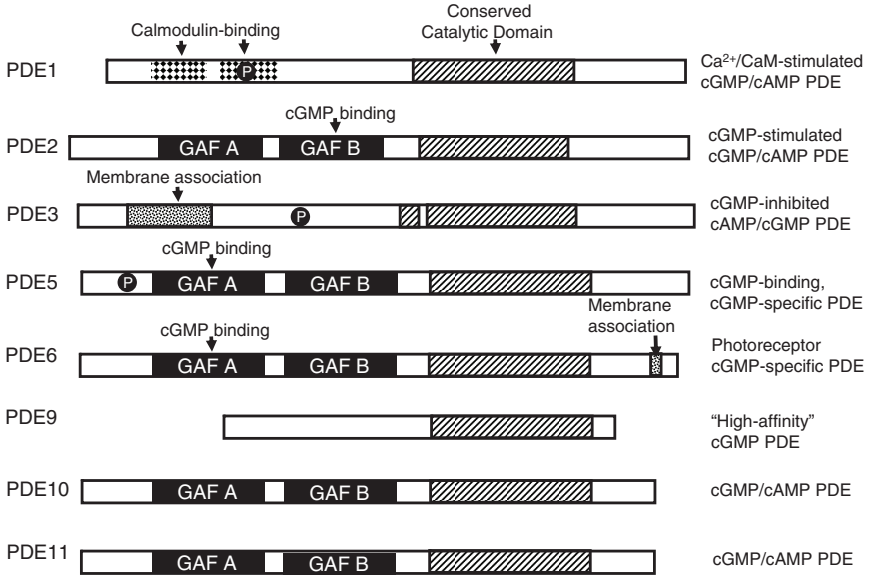

Fig. 1 Cyclic GMP-hydrolyzing PDEs. Eight of the eleven mammalian families of PDEs hydrolyze cGMP. Certain members of the PDE1 family (PDE1A and PDE1B), PDE5, PDE6, and PDE9 are selective for cGMP over cAMP whereas PDE1C, PDE2, PDE3, PDE10, and PDE11 hydrolyze both cGMP and cAMP well. The encircled P indicates phosphorylation sites and in the PDEs containing GAFs, these subdomains have been shown to provide for a variety of functions including allosteric cGMP binding (PDE2, PDE5, PDE6), dimerization contacts (PDE2, PDE5, PDE6), and regulation of the catalytic site (PDE2 and PDE5)

Table 1 Characteristics of cGMP-hydrolyzing phosphodiesterases

Phosphodiesterase	Descriptive name	Substrate K_m	(μM)	V_{max} μmol min^{-1} mg^{-1}
PDE1	Ca^{2+}/calmodulin-regulated PDE	cGMP cAMP	0.6–5.9 0.3–124	30–300 10–450
PDE2	cGMP-stimulated PDE	cGMP cAMP	10 30	123 120
PDE3	cGMP-inhibited cAMP PDE	cAMP cGMP	0.18–0.38 0.02–0.28	3–8.5 0.34–2
PDE5	cGMP-binding, cGMP-specific PDE	cGMP cAMP	2.5–6.2 290	1.3 1.0
PDE6	Photoreceptor PDE	cGMP cAMP	15–17 610–700	2300 –
PDE9	High-affinity cGMP PDE	cGMP cAMP	0.07–0.17 230	– –
PDE10	None	cGMP cAMP	13–14 0.22–1.1	– –
PDE11	None	cGMP cAMP	0.34–1.0[a] 0.45–1.6[a]	0.006–0.29[a] 0.009–2.9[a]

Range of values compiled from Bender and Beavo (2006)
[a] Weeks et al. (2007)
– No data available

PDEs are found in all cells and in almost all subcellular compartments; typically, a particular cell type contains PDEs from several families as well as variants from the same family. The pattern of expression of PDEs frequently varies with the developmental and proliferative status of the cell, the species in which the cell type is found, and the signaling exposures (e.g., hormonal stimuli) that the cell experiences (Barber et al. 2004; D'Sa et al. 2002; Jin and Conti 2002; Oki et al. 2000; Rybalkin et al. 1997; Shepherd et al. 2004; Takahashi et al. 2001; Yan et al. 1996). These variations are important considerations when drawing conclusions based on studies in one species and extrapolating that to other species. Another complication in understanding PDE functions is due to the fact that the catalytic properties of many PDEs overlap so that assignation of a specific role to a particular PDE family or to variants within a family is typically very challenging. Moreover, despite the considerable potential for overlap or duplication of functions of PDEs within a cell, this may or may not occur depending on subcellular compartmentation of PDEs (Fischmeister et al. 2005, 2006). Compartmentation accounts, at least in part, for creation of microdomains that spatially restrict cN diffusion and accentuate selectivity in targets altered by changes in cN levels (Baillie et al. 2003; Barnes et al. 2005; Conti et al. 2003; Houslay and Milligan 1997; Karpen and Rich 2001).

Among cells within an organism, excluding post-translational modification, it is now estimated that there are more than 100 PDEs. Some families e.g., PDEs 7–11, appear to be expressed sparsely and in only a few tissues, but others (PDE1, PDE2, PDE3, and PDE4 families) are widespread. With some exceptions, cellular concentration of PDEs is estimated to be fairly low since these PDEs have a high catalytic rate that is apparently sufficient for maintaining cN levels within the desired physiological range. However, in some cell types, the concentration of PDEs may be quite high, even higher than the cN levels themselves; retinal photoreceptor cells contain high concentrations (10–50 μM) of PDE6 (Cote 2006), and penile corpus cavernosum and lung contain an abundant amount of PDE5 (~0.1 μM) (Corbin et al. 2005; Gopal et al. 2001). The patterns of relative abundance can impose important restrictions on the action of PDE inhibitors. In some instances, the low level of a particular PDE in a tissue may still be important physiologically and pharmacologically.

1.2 PDEs as Drug Targets

1.2.1 Evolution and Mechanism of Action of PDE Inhibitors

In the early years, Sutherland and colleagues determined that caffeine and theophylline compete with cAMP or cGMP for access to the catalytic sites of PDEs; even today, most PDE inhibitors are known to be competitive. Caffeine and theophylline contain a purine similar to the adenine and guanine of cAMP or cGMP, respectively, and are believed to compete for similar contacts in PDEs. The early identification of effective inhibitors of PDE action provided a powerful tool for investigating cN-signaling pathways and laid the groundwork for design and synthesis of progressively more potent and selective PDE inhibitors, many of which had molecular structures closely related to those of cNs and caffeine. In the 1960s, caffeine and closely related compounds such as theophylline were the only reliable, albeit weak, inhibitors of PDEs. A synthetic inhibitor, 3-isobutyl-1-methylxanthine (IBMX), which has a similar structure to caffeine and theophylline, became available in the early 1970s and has been widely used to block PDE action in cells as well as in in vitro studies.

By the 1980s and into the 1990s, researchers had mapped the spatial features of numerous PDE catalytic sites using a collection of cN analogs (Beltman et al. 1995; Butt et al. 1995; Erneux et al. 1984; Francis et al. 1990; Sekhar et al. 1996; Wada et al. 1987). Beginning in 2000, success in obtaining the x-ray crystal structures of numerous isolated C domains has profoundly advanced development of new PDE inhibitors. Insights derived from these many lines of investigation provide the basis for rational design and synthesis of inhibitors that are progressively more potent and selective for particular PDE families.

1.2.2 Selectivity of PDE Inhibitors

Even with such a wealth of structural information, the design of potent and selective inhibitors is still problematic. Medicinal chemists continue to be challenged to design inhibitors that are potent and highly selective for particular PDE families, inhibitors that are selective for variants within a family, inhibitors that do not interact significantly with proteins other than PDEs (Ke and Wang 2006; Zhang 2006) and inhibitors that possess the necessary pharmacokinetic and safety properties to be used as a medicinal agent. Medicinal and investigational use of PDE inhibitors has frequently encountered interpretational problems due to interaction of PDE inhibitors with non-PDE proteins. Theophylline, which is used in a number of medications, has long been known to cause some of its therapeutic effects through interaction with adenosine receptors as well as by inhibition of PDE activity. The selective PDE2 inhibitor, erythro-9-(2-hydroxy-3-nonly)adenine (EHNA) with $IC_{50} = 0.8\,\mu M$, is more potent as an inhibitor of adenosine deaminase ($IC_{50} = 1\,nM$) (Podzuweit et al. 1995). More recently, zaprinast, long touted as a selective inhibitor of cGMP-hydrolyzing PDEs such as PDE5, PDE6, and to some extent PDE1, has been shown to be a potent agonist for the orphan receptor, GPR-35 (Guo et al. 2008; Taniguchi et al. 2006), and its effects in some cells are apparently related to changes in calcium homeostasis, but not to cGMP. These complications are not surprising since many inhibitors contain a purine-like component that is found in many nucleotides. For this reason, the use of a collection of PDE inhibitors in studying a biological process is strongly advised in order to verify that the observed effect follows the pattern of inhibitor potency for a particular PDE.

Assignation of a value for selectivity of an inhibitor among PDE families is still fraught with problems that require more careful consideration (Bischoff 2004). The term "specific" rarely applies to a PDE inhibitor including the inhibitors currently referred to as "PDE5-specific inhibitors." The term "specific" is commonly considered applicable to compounds having a >50-fold selectivity for one PDE family versus other PDEs. However, using inhibitors of PDE5 that are commonly referred to as "selective" (zaprinast, sildenafil, vardenafil, and tadalafil) as an example, one can see that these inhibitors do not meet this criterion. Zaprinast potency for inhibition of PDE6 is ~10-fold greater than the potency for inhibition of PDE5. Furthermore, sildenafil and vardenafil inhibit isoforms of PDE6 with only 3–10-fold lower potency than PDE5 (Zhang et al. 2005b), and tadalafil inhibits isoforms of PDE11 with 7–45-fold lower potency than that for PDE5 depending on the length of the construct (Weeks et al. 2007).

The problems with assessing and assigning selectivity of PDE inhibitors arise from many factors; screening for potency and selectivity is commonly performed with one isoform of the respective PDE families, perhaps with a construct containing only the catalytic domain (C domain), and with little or no consideration for the effect of regulatory features on affinity for the inhibitor. An example of the problem is revealed when considering the impact of regulatory features and structural features of PDE5 on affinity for inhibitors described below. Potencies of inhibitors for PDE5 are significantly enhanced by allosteric cGMP binding, phosphorylation, prolonged exposure to inhibitors, and, in the case of vardenafil, by the physical presence

of a portion of the regulatory domain containing GAF B (Bessay et al. 2008; Blount et al. 2006, 2007; Francis et al. 2006). Since "selectivity" of these inhibitors for PDE5 has been assessed using enzyme that is not altered by these processes, it is possible that the degree of selectivity versus other PDEs has been significantly underestimated. Of course, this assumes that the affinities of other PDEs for these inhibitors are not altered by their own regulatory processes. Furthermore, important leads for development of more potent inhibitors may be missed by screening inhibitors using isolated C domains; in the isolated C domain of PDE5, vardenafil is equipotent with sildenafil and tadalafil but vardenafil potency is 10- to 40-times greater in PDE5 holoenzyme (Blount et al. 2006).

Interpretation of studies using "selective" PDE inhibitors requires considerable caution. In platelets, evidence suggests that PDE5 inhibitors cause an increase in the cGMP level which then competes with cAMP for the catalytic site of PDE3 (also known as the cGMP-inhibited cAMP-PDE). This competition spares cAMP thereby fostering its elevation to bring about observed anti-aggregatory effects (Maurice 2005; Maurice et al. 1991). Thus, cGMP, a natural substrate for PDE3, also acts as an inhibitor for PDE3 to foster accumulation of cAMP. This same scenario is plausible for any dual-specificity PDE since elevation of either cGMP or cAMP would allow the other nucleotide to accumulate and increase signaling through that pathway. For these PDEs (PDE1, PDE2, PDE3, PDE10, and PDE11), either cN can act as a competitive "inhibitor"; thus any of these enzymes could be described as a "cN-inhibited" PDE. This emphasizes that effects of blocking the action of one PDE family must be viewed within the complex context of the population of PDEs and other cN-binding proteins in a particular cellular milieu.

1.3 Characteristics of PDEs

1.3.1 Domain Structure of PDEs

PDEs are comprised of multiple domains and subdomains including the conserved C domain, which is typically located toward the C-terminus of the protein, and N-terminal and extreme C-terminal regions that are more structurally diverse (Fig. 1) (Burns et al. 1996; Charbonneau et al. 1986; Francis et al. 2001; Houslay and Adams 2003; Mehats et al. 2002; Soderling and Beavo 2000). In many instances, the N-terminal region provides regulatory features for modulation of catalytic activity and can accurately be described as a regulatory domain (R domain, a descriptor that applies to PDE1, PDE2, PDE3, PDE4, PDE5, PDE6, and PDE11). However, in other PDEs, a role of this region in enzyme function has not been established. Furthermore, some regulatory functions reside in the C-terminal region of the enzymes (Baillie et al. 2001; Hoffmann et al. 1999). Association of PDEs with other proteins or cellular membranes can be mediated by either the N-terminal or C-terminal region, e.g., PDE6 is tethered to membrane structures by covalent modification at a –CAAX– motif at the extreme C-terminus (Cote 2006), and subcellular localization

of members of the PDE3 and PDE4 families is mediated by components in their N-terminal region (Beard et al. 2002; Shakur et al. 2000).

The salient features of PDE catalytic action are preserved in many isolated C domains (Fink et al. 1999). Currently, x-ray crystal structure of one PDE holoenzyme (PDE2) has been determined (J. Pandit, Pfizer Global Research & Development, personal communication), whereas structures of several only the isolated R domains or isolated C domains have been determined (Huai et al. 2004; Martinez et al. 2005; Scapin et al. 2004; Sung et al. 2003; Xu et al. 2000, 2004; Zhang 2006). The impact of structural and functional changes that derive from oligomerization or other contacts unique to PDE holoenzymes should not be underestimated. These differences can impact both catalytic and regulatory function of PDEs, including interactions with PDE inhibitors (Bessay et al. 2007; Blount et al. 2006, 2007; Wang et al. 2008; Weeks et al. 2007).

1.3.2 Factors that Provide for Catalysis and Inhibitor Action

Breakdown of cN by PDEs occurs within a deep hydrophobic pocket in the C domain; the catalytic site is formed by juxtaposition of amino acids from three distal segments of the C domain. While there are differences in the molecular features of PDE catalytic sites, all x-ray crystal structures thus far support a general profile proposed for interaction with cN (Ke and Wang 2006; Zhang et al. 2004; Zhang 2006). Two structural components of cNs provide key contacts with the PDE catalytic site, i.e., the purine ring, either guanine or adenine, and the novel cyclic phosphate ring. However, it must be noted that to date there is no crystal structure of a cN in complex with a PDE catalytic site so that our current understanding of these interactions is based on modeling. Among PDEs, the rates of catalytic breakdown of cN and the affinities of the enzymes for cN substrates vary widely; affinities of different PDEs for cN substrates differ 1,000-fold. Even within a single family such as PDE1, affinity for a single cN, e.g., cAMP, can vary 100-fold. Maximum catalytic rates, i.e., V_{max} values, among different PDEs also cover a broad range ($\sim$0.1–150 μmol min^{-1} mg^{-1}) (Bender and Beavo 2006). This emphasizes that although PDE C domains share many common features, each individual catalytic site has characteristics that alter the efficacy with which the cyclic phosphate ring is broken and also dictate the specific spatial and chemical features that foster interaction with the purine. These distinctions among the sites provide the basis for designing more potent and more selective inhibitors.

Divalent cations are required to support PDE catalytic activity, and a critical role for Zn^{2+} in the catalytic process of PDEs was first demonstrated for PDE5 (Francis et al. 1994, 2000; Omburo et al. 1995). The molecular determinants that provide for effects of divalent cations have been documented by site-directed mutagenesis in a number of PDEs and are now more precisely revealed in the x-ray crystal structures of PDE C domains. All of these structures contain two divalent metal-binding sites that are closely approximated; one site is most commonly occupied by a tightly-bound Zn^{2+}. The metal occupying the second site is not yet identified, but it is generally assumed to be either Mg^{2+} or Mn^{2+}. Under physiological conditions,

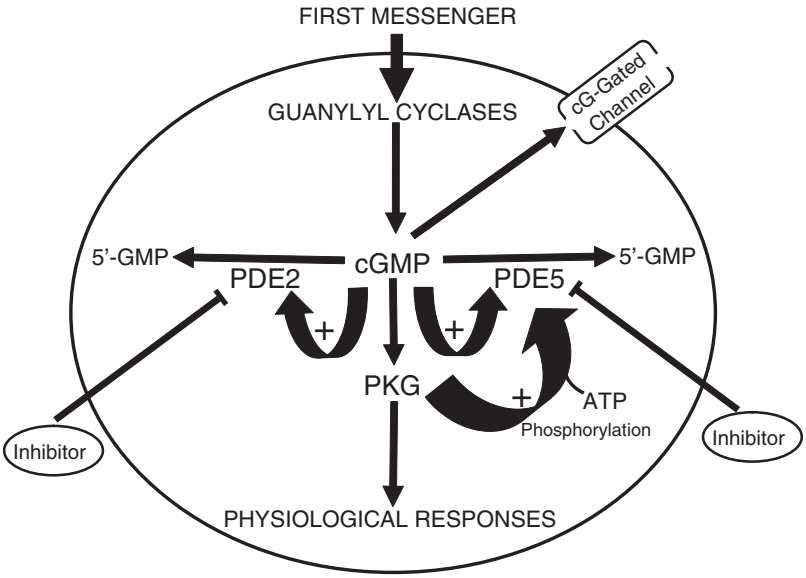

Fig. 2 Negative feedback regulation of cGMP-signaling through PDE2 and PDE5. Intracellular level of cGMP is determined by the balance between the rate of synthesis by guanylyl cyclases and breakdown by PDEs. Synthesis is increased when first messenger molecules such as natriuretic peptides, and nitric oxide stimulate the catalytic activity of the cyclases. PDE2 and PDE5 bind cGMP at regulatory sites in their respective R domains, and this interaction increases the affinity of these enzymes for cGMP at their catalytic sites, increased breakdown of cGMP, and negative feedback regulation. In addition, cGMP binds to and activates PKG resulting in phosporylation of cellular proteins that bring about physiological effects of cGMP. PKG phosphorylaes PDE5 thereby increasing PDE5 affinity for cGMP, greater cGMP breakdown, and negative feedback regulation of the cGMP-signaling pathway

these sites are presumed to be saturated, but at least for PDE4, the sensitivity of the enzyme to Mg^{2+} in vitro is affected by regulatory processes (Laliberte et al. 2000, 2002; Lim et al. 1999; Oki et al. 2000; Sette and Conti 1996). A hydroxyl derived from a highly polarized water molecule that bridges the metals is proposed to act as the nucleophilic catalyst to break a phosphorous-oxygen bond in the cyclic phosphate ring (Fig. 2). This hypothesis is in agreement with elegant biochemical studies and mechanistic interpretations derived from early studies using $H_{218}O$ and cN analogs (Burgers et al. 1979; Goldberg et al. 1980; Jarvest et al. 1982). For PDE5, the rate-limiting step of catalysisn has been determined to be the hydrolytic reaction (Morris, Z., Francis, S., Corbin, J., unpublished results). Variation in the rates of cN hydrolysis among PDE families may be in part due to different complements of divalent cations in the catalytic site and/or different efficiencies in polarizing the catalytic hydroxyl nucleophile. Inhibitors that interact with the catalytic site metal ions may prove to be particularly potent.

The selectivity and affinity of PDEs for cAMP or cGMP is determined by direct contact between certain C domain residues with particular components of the

respective purines and cyclic phosphate rings, and many of these contacts are also exploited for inhibitor binding. Sequences outside the immediate environs of the catalytic site can also differentially impact PDE affinity for substrate and inhibitors; this is demonstrated by the effect of phosphorylation to improve affinity of PDE5 for substrates or inhibitors (Bessay et al. 2008; Corbin et al. 2000), the selective effect of mutations of the H-loop of PDE5 on affinity for substrate versus that for sildenafil (Wang et al. 2006), and the selective effect of GAF B of PDE5 on affinity for vardenafil versus that for sildenafil, tadalafil, and cGMP (Blount et al. 2006).

In the high-resolution x-ray crystal structures of PDE C domains, three important sets of contacts appear to be crucial for interaction with cN substrate or inhibitors; the veracity of these predicted contacts for cN substrate is limited since they are based on modeling of low-affinity product in the catalytic sites. Within these limitations, important contacts include: (1) an invariant glutamine, (2) hydrophobic interactions in which the purine is "clamped" between hydrophobic residues, and (3) charge interactions that apparently coordinate the cyclic phosphate moiety through contacts with the divalent metals and surrounding amino acids. Most of these contacts are conserved among PDE families, which set limits for PDE inhibitor selectivities based on these interactions; in numerous cases, PDE inhibitor selectivities result from contacts involving non-conserved amino acids, many of which lie just outside the conserved catalytic site of PDEs. Potencies of inhibitors for each PDE are also conferred in part by contacts made with non-conserved amino acids that lie outside the conserved pocket. In other words, the natural substrate cGMP (or cAMP) makes contacts with amino acids in the conserved central pocket, but inhibitors make contact with non-conserved amino acids outside this pocket, in addition to the amino acids in the conserved central pocket.

1.3.3 Regulation of PDE Activities

Short-term regulation of PDE activities can be mediated by a complex group of processes including phosphorylation/dephosphorylation, binding of small ligands such as cGMP or phosphatidic acid to allosteric sites, translocation between subcellular compartments, and association with selected protein inhibitors or activators. Many of these processes are involved in negative feedback regulation of cN levels, a process commonly found in other biochemical pathways. In addition, there are long-term regulatory mechanisms including increased production of PDEs in response to a chronic stimulus to elevate cN. A detailed discussion of factors that impact and regulate the activity of each PDE family is beyond the scope of this chapter, but several general themes are worthy of mention.

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In most tissues, cellular cN levels rarely reach high levels ($>10\,\mu M$) even when confined to specific subcellular compartments. If that is so and considering the K_m of most PDEs for cN substrates, the rate at which PDEs function catalytically would typically be less than maximal. Consequently, when cellular cN rises, hydrolytic

activity by PDEs would increase proportionately, i.e., until the maximum catalytic rate (V_{max}) is reached; this is simply a process of mass action, i.e., the greater the concentration of the substrate, the faster the rate until the catalytic rate can increase no further. Aside from the change in substrate supply, a number of PDE families have been shown to undergo reversible activation and inactivation by a variety of processes. Most PDEs that have been studied exist in at least two states of catalytic activity, i.e., a less active conformer and a more active conformer; for PDE5, the more active form of the enzyme appears to assume a more elongated shape. Transition between activity states can be facilitated by events that modulate an intrinsic autoinhibition derived from influences within the PDE protein itself, or in some cases, by inhibitory influences imposed by regulatory proteins such as the inhibitory Pγ proteins that bind to PDE6 in photoreceptor cells, or influences provided by activating proteins such as calmodulin that binds to and activates PDE1. Relief of inhibitory influences can increase catalytic rate (V_{max}) as occurs in the PDE4 and PDE6 families or increase in affinity (K_m) for cN substrate as occurs in activation of PDE2, PDE3, and PDE5. While these physically and kinetically distinct forms of PDEs are commonly interconvertible, they may also exhibit considerable stability (Francis et al. 1998), (Corbin et al., unpublished results).

Catalytic sites of some PDEs exhibit a plasticity that manifests as a substantially altered affinity for inhibitor. Interaction of PDE4 with the inhibitor rolipram is the best characterized example of this phenomenon. Two populations of PDE4 isoforms exhibit either "low-affinity rolipram-binding sites" (binding affinities in the range of 0.1–1 μM) or "high-affinity rolipram-binding sites" (binding affinities in the range of 1–50 nM); both populations can exist in the same tissue, or one form may predominate (Houslay and Adams 2003; Laliberte et al. 2000; Liu et al. 2001). Results support the interpretation that these affinity states are relatively stable and reflect different conformers of a single catalytic site on PDE4. This suggests that contacts within the catalytic site can be adjusted and refined in a manner that optimizes interaction with the inhibitor. There is now evidence that at least one other family, i.e., PDE5, exhibits similar heterogeneity and plasticity that impacts the potency and selectivity of inhibitors (Blount et al. 2004; Corbin et al. 2003).

2 Negative Feedback of cGMP Signaling Mediated by cGMP-Hydrolyzing PDEs

PDE2, PDE3, and PDE5 are directly involved in negative feedback regulation of cN-signaling pathways, and PDE1 may play a similar role. It is predicted that inhibitors that are developed for most PDE families will not only raise tissue cAMP or cGMP but, by the fact that the inhibitors occupy the PDE catalytic site, they will block a negative feedback process involving the targeted PDE as well, thus potentiating inhibitor efficacy. Cyclic GMP-PDEs and negative feedback will be discussed herein.

2.1 Regulation of PDE2

PDE2 hydrolyzes cGMP and cAMP with approximately equal affinity and efficacy and also binds cNs at allosteric sites; it is sometimes described as mediating "cross-talk" between cGMP- and cAMP-signaling pathways. PDE2 is commonly known as the "cGMP-stimulated cAMP PDE," a somewhat misleading moniker since PDE5, at least, is also a cGMP-stimulated PDE. Moreover, occupation of the PDE2 allosteric site by either cGMP or cAMP enhances its catalytic rate (Martinez 2006). The affinity of the allosteric site for cGMP is ~10-fold greater than for cAMP, but cAMP level in cells is typically much higher than cGMP so that cAMP has significant potential to occupy the PDE2 allosteric site to accelerate cN breakdown (Wu et al. 2004). Therefore, it seems likely that interaction of cAMP or cGMP with the allosteric site could foster increased activity at the catalytic site to break down cNs, thereby mediating negative feedback and/or cross-talk for the signaling pathways. When cGMP levels are elevated in adrenal glomerulosa cells in response to atrial natiuretic peptide, cGMP binds to allosteric sites on PDE2 and activates the enzyme to increase breakdown of cAMP, thereby lowering aldosterone production; this is a good example of a PDE mediating cross-talk between the cN-signaling pathways (MacFarland et al. 1991). However, in cardiomyocytes, PDE2 controls cGMP level in regions near the plasma membrane, whereas PDE5 controls cGMP levels in the cytosol (Castro et al. 2006); in both instances, elevation of cGMP in that portion of the cell would be predicted to increase cGMP binding to the allosteric sites of the two PDEs resulting in increased activity at the catalytic site and activation of a negative feedback pathway (Fig. 2). For PDE5 this scenario has also been demonstrated in platelets and in vascular smooth muscle cells (SMC). Since the catalytic region of PDE2 hydrolyzes both cAMP and cGMP with roughly equal efficacy, PDE2 may be involved in cross-talk between the signaling pathways or provide for negative feedback regulation of both the cGMP- and cAMP-signaling pathways.

2.2 Regulation of PDE3

Elevation of cAMP in liver by hormones such as glucagon or epinephrine stimulates glycogen breakdown and gluconeogenesis, resulting in increased glucose output. This is an important survival advantage during starvation. Elevated cAMP activates PKA, which phosphorylates and activates PDE3B. Activated PDE3 increases cAMP degradation, thereby mediating classical negative feedback control of the cAMP-signaling pathway (Corbin et al. 1985; Degerman et al. 1990). PDE3B mediates a similar negative feedback process for epinephrine regulation of lipolysis in adipose tissue. PDE3 degrades cGMP as well as cAMP, but whether PDE3B is phosphorylated and activated by PKG to mediate negative feedback for cGMP signaling is not clear. Inhibition of PDE3 hydrolysis of cAMP by cGMP is also implicated in mediating cross-talk between the cAMP- and cGMP-signaling pathways (Maurice 2003).

2.3 Regulation of PDE1

PDE1 is activated by binding Ca^{+2}/calmodulin to its R domain (Sonnenburg et al. 1995). In some tissues, e.g., heart, it is thought that cAMP elevation by epinephrine or sympathetic nerves increases free Ca^{+2} in contractile cells. This Ca^{+2} not only increases the contractile force of the heart and produces other effects, it simultaneously activates PDE1 to increase cAMP degradation, which represents yet another negative feedback process involving PDEs.

2.4 Regulation of PDE5

2.4.1 General Features of PDE5 That Contribute to Catalytic Site Function

PDE5 catalytic activity is regulated by myriad mechanisms that contribute importantly to negative feedback regulation of cGMP signaling and are quite complex (Corbin and Francis 1999; Corbin et al. 2000; Mullershausen et al. 2003; Rybalkin et al. 2003). Until recently, PDE5 was known as the cGMP-binding cGMP-specific PDE because it contains allosteric sites and a catalytic site that are both highly specific for cGMP (Fig. 3). PDE5 is a modular protein with a more N-terminal R domain and a more C-terminal C domain (Fig. 3) (McAllister-Lucas et al. 1993). There is significant communication between the PDE5 R domain and C domain, and this property fashions a complex and well integrated modulation of enzyme activity. The cGMP-binding sites in the R domain and the catalytic site are not evolutionarily related, but both exhibit ~100-fold specificity for cGMP versus cAMP.

When the C domain alone is generated as a recombinant protein, it is a monomer that retains the salient features of catalysis exhibited by PDE5 holoenzyme (Fink et al. 1999; Sung et al. 2003). PDE5 exhibits a K_m for cGMP of ~2–3 μM, a concentration that is generally considered to be well above normal physiological concentration range for cGMP. Comprehensive studies using site-directed mutagenesis of conserved amino acids in the C domain of PDE5 first identified residues that are important for contact with the critical divalent cations, those that contribute to binding of the substrate, and many of those that are key to potent interaction of inhibitors such as sildenafil, vardenafil, and tadalafil (Corbin et al. 2006; Turko et al. 1998, 1999; Zoraghi 2007).

2.4.2 Features of the PDE5 Regulatory Domain

The N-terminal R domain of PDE5 contains a number of functional sub-domains that impact PDE5 catalytic function and its interaction with inhibitors. The sub-domains include two GAFs (A and B), a single phosphorylation site (Ser-102) that is preferentially phosphorylated by PKG, and dimerization contacts (McAllister-Lucas et al. 1993). High-affinity allosteric cGMP binding is provided by the GAF

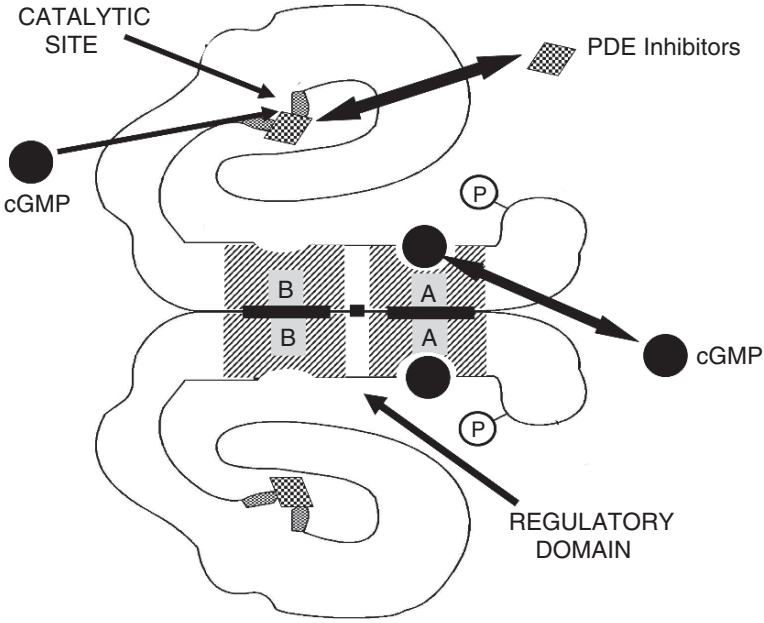


Fig. 3 Working model of PDE5. PDE5 is a dimer of two identical ~100-kDa subunits. It has a C domain located in the more carboxyl-terminal portion of the protein and a R domain located in the more amino-terminal portion. Dimerization occurs through interactions at multiple points in the regulatory domain indicated by the black bars in the regulatory domain. The catalytic machinery is depicted as two ovals that reflect the two divalent cations that provide for catalytic hydrolysis of cGMP. Cyclic GMP is also bound to allosteric sites in the R domain including a high-affinity site in GAF A (indicated by the deep oval binding pocket) and perhaps in GAF B; GAF B has been shown to have numerous regulatory functions in PDE5 and may have weak cGMP-binding activity as indicated by the shallow oval pocket. PDE5 can be specifically phosphorylated by PKG at a single site, Ser-102, in the R domain, and exposure of this serine is regulated by binding of cGMP and other ligands to the protein

A sub-domain (Rybalkin et al. 2002; Zoraghi et al. 2005), but whether GAF B can also bind cGMP is not known. In the holoenzyme, binding of cGMP to the allosteric cGMP-binding sites is low, but occupation of the catalytic site by either cGMP or PDE5 inhibitors stimulates cGMP binding to the allosteric sites (Bessay et al. 2007; Thomas et al. 1990). Cyclic GMP occupation of the allosteric sites increases affinity of the catalytic site for cGMP and inhibitors. Furthermore, when the allosteric sites are occupied by cGMP, PDE5 undergoes a conformational change that exposes Ser-102 for rapid phosphorylation by PKG (Thomas et al. 1990). Phosphorylation of Ser-102 in turn increases the affinity of the allosteric cGMP-binding sites for cGMP as well as affinity of the catalytic site for substrate or inhibitors (Bessay et al. 2008; Corbin et al. 2000).

2.4.3 Conformational States of PDE5

Multiple lines of evidence suggest that PDE5 exists in at least two conformations and that the distribution of the PDE5 population between these conformers can be regulated by phosphorylation/dephosphorylation, oxidation/reduction, and binding of cGMP or inhibitors. These stepwise modulations of structure and function in PDE5 are tightly controlled and regimented and bear significant consequences for feedback regulation of cGMP levels and inhibitor potency. First of all, cGMP occupation of the allosteric cGMP-binding site increases the affinity of the catalytic site for cGMP, the substrate, or inhibitors such as sildenafil, vardenafil, or tadalafil. In the absence of PDE5 inhibitors, the rate of hydrolysis of cGMP is increased since the PDE5 catalytic site is usually not saturated with cGMP; with higher affinity of the catalytic site for cGMP, more will be bound to the site and hydrolyzed. As a substrate mimic, a similar event occurs for PDE5 inhibitors, i.e., cGMP binding to the allosteric sites increases the affinity for the inhibitor (Blount et al. 2006). However, the inhibitor is not degraded by PDE5 and will remain bound to the catalytic site for a longer period of time, thus representing a feed-forward process that enhances inhibitor potency. Phosphorylation of Ser-102 increases allosteric cGMP-binding as well as affinity of the catalytic site for cGMP or inhibitors, thereby fostering improved function of the catalytic site.

2.4.4 Integration of PDE5 Regulatory Processes in Negative Feedback Control of cGMP Level

The physiological and pharmacological impacts of the regulatory processes described above are clear and are likely to occur in many tissues. For example, when cGMP is elevated in vascular smooth muscle, as occurs in the penile corpus cavernosum during sexual arousal, these regulatory factors, including increased allosteric cGMP binding and phosphorylation of Ser-102, impact PDE5 function and the physiological process associated with the vasodilation that leads to penile erection. Activation of guanylyl cyclases to produce more cGMP will increase the activity of the catalytic site in at least three ways to degrade cGMP (Fig. 3). First, elevation of cGMP will cause more degradation of cGMP, simply due to a greater supply of cGMP to the PDE5 catalytic site. Second, increased cytosolic cGMP will foster cGMP binding to the allosteric cGMP-binding sites of PDE5, and this will further increase hydrolysis of cGMP at the catalytic site. Third, phosphorylation of Ser-102 on PDE5 will further increase the affinity of allosteric cGMP binding and foster greater catalytic activity. All of these effects act to dampen or terminate the cGMP signaling initiated by sexual arousal and act in concert as a negative feedback regulatory process. The increased affinity of the enzyme for substrate would also provide for improved competition with inhibitors that act at the catalytic site as well, thereby blunting the potency of inhibitors to some extent. However, in a physiological setting, effects of potent PDE5 inhibitors prevail since cGMP accumulates, thereby providing for vasodilation sufficient for improved penile tumescence.

2.4.5 Sequestration of Cellular cGMP by PDE5

Negative feedback control of the cGMP pathway through sequestration by PDE5 could occur in some cells. In the corpus cavernosum, the PDE5 allosteric cGMP-binding GAF A is >100 nM (Gopal et al. 2001), which is higher than the concentration of cGMP (basal ~20 nM). Most of this cGMP would be tightly bound to GAF A and unavailable for its target receptors such as PKG. Increased sequestration by this process would be expected to occur after cGMP elevation and stimulation of the other negative feedback systems described above. Whether or not PDE2 concentration is high enough in most cells to mediate significant sequestration of cGMP, or cAMP, has not been demonstrated. PDE6 concentration in retinal photoreceptors cells is clearly high enough to impart sequestration (Cote 2006).

2.4.6 Integration of PDE5 Regulatory Processes to Increase Potency of Inhibitors and Enhance Efficacy of Action

Some of the same sequence of events described as providing for negative feedback regulation of cGMP level can also enhance the potency and efficacy of PDE inhibitors. PDE5 inhibitors occupy the catalytic site of PDE5 to block cGMP access and thereby inhibit cGMP degradation. As a result, cGMP is readily available to bind to PDE5 allosteric cGMP-binding sites, bind to and activate PKG, and foster phosphorylation of Ser-102 on PDE5 by PKG, which enhances inhibitor binding at the catalytic site and increases the cGMP level further (Fig. 3). Increased cGMP binding to the allosteric sites in turn promotes tighter binding of inhibitors at the catalytic site which elevates cGMP even more. In the presence of inhibitor, this regulatory process becomes a feed-forward process to promote potency and efficacy of the pharmacological action of competitive inhibitors. Furthermore, prolonged exposure of PDE5 to inhibitors causes the enzyme to undergo a conformational change that increases the affinity of the enzyme for the inhibitor. Altogether, these effects that in nature provide a powerful counter to cGMP elevation in tissues containing PDE5 also provide a powerful feed-forward process to improve potency and action of PDE5 inhibitors.

3 Inhibitors of cGMP-Specific PDEs

3.1 Inhibitors of PDE5

3.1.1 Structures, Selectivities, and Potencies of PDE5 Inhibitors

Naturally occurring weak and nonselective PDE inhibitors such as caffeine or theophyline, which are used for the treatment of asthma, and the natural substrate of PDE5, cGMP, served as starting points for the development of more potent and

selective synthetic PDE5 inhibitors (Fig. 4). IBMX, with PDE5 inhibitory potency in the micromolar range, was the prototype of many structurally similar PDE inhibitors, but it has little or no selectivity for a panel of other PDEs. Zaprinast (MB 22948) set a milestone in the evolution of more potent PDE5 inhibitors. It was originally designed in an effort to prepare xanthine derivatives for selective inhibition of PDEs that preferentially hydrolyzed cGMP and for use in treatment of allergic asthma (Broughton et al. 1974). Its effectiveness in inhibiting cGMP-selective PDEs was initially shown in crude extracts (Frossard et al. 1981a, b); it was later shown to be particularly potent for PDE5 and PDE6 (Frossard et al. 1981a, b; Lugnier et al. 1986; Turko et al. 1999; Zhang et al. 2005b). Zaprinast has been widely used to inhibit PDEs selective for cGMP and to establish the important role of cGMP as a second messenger in SMC; stronger selectivity of zaprinast inhibition of PDE5 compared to PDE1 came to be appreciated in the late 1980s (Beavo and Reifsnyder 1990), but it proved to be an important pharmacologic tool for the evaluation of the effect of inhibition of cGMP-selective PDEs, including PDE5, in various conditions including models for the signaling mechanism in erectile function (Bush et al. 1992; Rajfer et al. 1992; Trigo-Rocha et al. 1993).

The focus of potent and also selective PDE5 inhibitors that were disclosed in patents during the late eighties and early nineties was their use in cardiovascular indications. This was also true for sildenafil whose structure was first published in 1992 (Fig. 4). The successful development of sildenafil as the first orally active and highly efficient drug for the treatment of erectile dysfunction (ED) made this structure the most widely recognized PDE5 inhibitor. Two other PDE5 inhibitors, vardenafil (Haning et al. 2002) and tadalafil (Padma-Nathan et al. 2001; Rotella

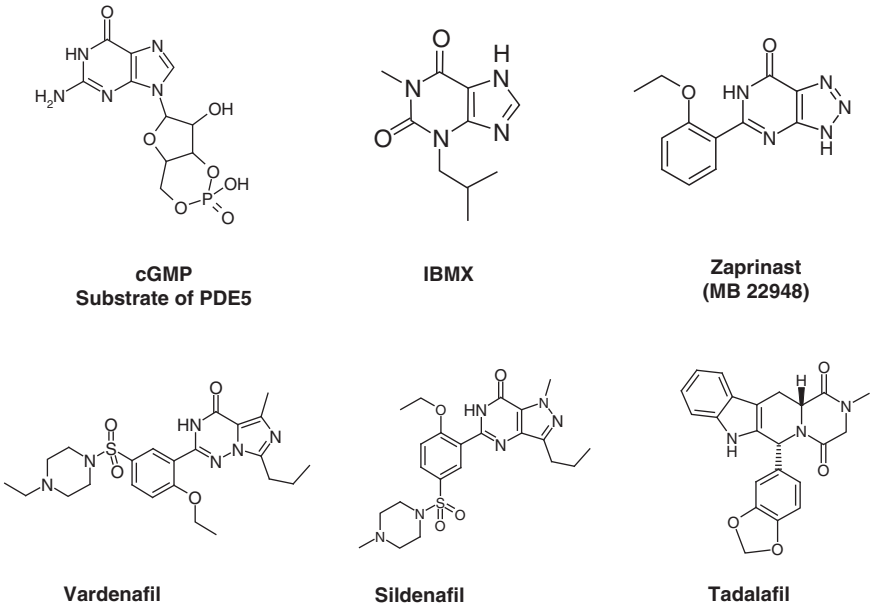


Fig. 4 Structural relationships of PDE5 inhibitors

2003) entered the market in 2003. Sildenafil as well as vardenafil are analogs of the PDE5 substrate. The core structure of sildenafil is a pyrazolopyrimidinone heterocyclic system (Haning et al. 2003). Vardenafil is distinguished by its imidazotriazinone core structure which has impact on its increased potency (Corbin et al. 2004; Haning et al. 2003). Despite the structural similarities of sildenafil and vardenafil (Fig. 4), the two compounds differ substantially in distribution of electronegativity in the heterocyclic rings (Corbin et al. 2006). While the overall binding characteristics of sildenafil and vardenafil to the PDE5 catalytic site are reported to be similar (Sung et al. 2003), significant differences have been revealed in subsequent x-ray crystal structures of PDE5 C domain in complex with these inhibitors (Wang et al. 2008). These structures reveal that (a) the conformation of the H-loop differs, (b) the molecular configuration of bound vardenafil differs from that of sildenafil, and (c) unlike sildenafil, vardenafil causes loss of the divalent cations from the PDE5 catalytic site (Wang et al. 2008). Other potent PDE5 inhibitors (udenafil, mirodenafil, SLX-2101, dasantafil, and avanafil) have recently been reported, but full information on the degree of selectivity of these compounds is currently not available (Fig. 5). Tadalafil is an indole-type PDE5 inhibitor with an improved selectivity against PDE6 compared to sildenafil and vardenafil but lower selectivity for PDE11 (Card et al. 2005; Weeks et al. 2005) (Fig. 4).

X-ray crystallographic analysis of PDE5, together with structurally different PDE5 inhibitors as well as binding studies of PDE5 inhibitors to genetically modified PDE5 enzymes, has provided new insight into factors that influence PDE5 activity. The availability of the first three commercial drugs was not only a revolution in therapy of ED, but it was also tremendously stimulating for research, resulting

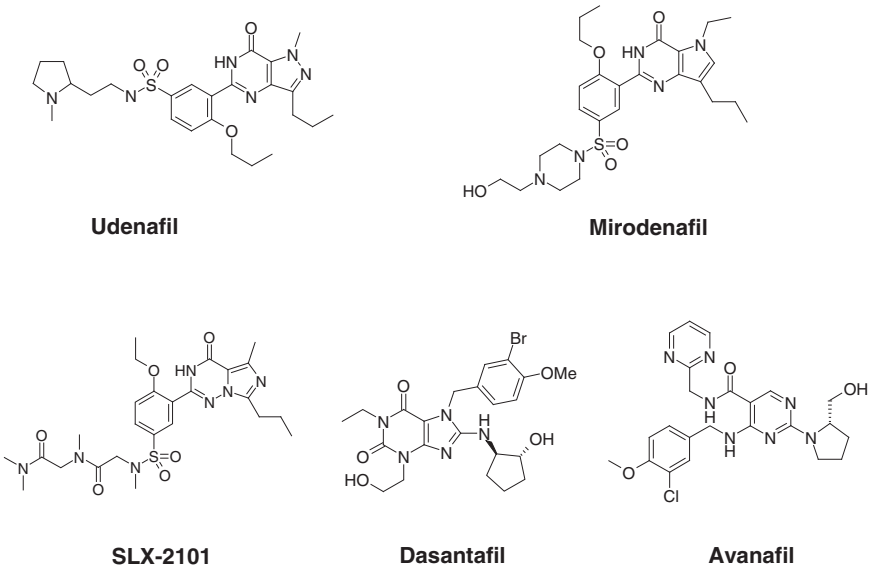


Fig. 5 New PDE5 inhibitors

in a deeper understanding of cGMP-controlled pathways. The wide and safe use of PDE5 inhibitors has triggered a number of attempts to find new applications for these agents. The approval of sildenafil for treatment of pulmonary arterial hypertension (PAH) is one striking example. A variety of other potential new indications for PDE5 inhibitors are on the horizon and are discussed below. Potency and selectivity of the commercially available PDE5 inhibitors are very high as well as the therapeutic standard of ED treatment based on the mechanism of PDE5 inhibition. However, there are still intense research and development efforts ongoing to discover new PDE5 inhibitors. Two of them, udenafil (Kim et al. 2005) and mirodenafil (SK Chemicals 2001) (Fig. 5) have recently entered the Korean market for the treatment of ED. Other compounds with improved potency and/or pharmacokinetics could be candidates for development in new indications.

Chemistry and structure activity relation (SAR) of PDE5 inhibitors has been extensively reviewed (Haning et al. 2003; Hendrix and Kallus 2004; Rotella 2002; Sandner et al. 2008). Taken together, PDE5 inhibitors are an exciting class of drugs with a unique mechanism of action combined with a favorable safety profile. Intense preclinical and clinical research with PDE5 inhibitors has revealed new mechanisms and a number of potential new indications for these drugs. It is envisaged that the PDE5 inhibitors may become valuable future treatment options for a variety of diseases beyond ED and pulmonary hypertension.

3.1.2 Pharmacology of PDE5 Inhibition

It is well established that NO and natriuretic peptides increase intracellular cGMP level by stimulation of the NO-sensitive and membrane-bound guanylyl cyclases, respectively. This cGMP formation is the initial step of a biochemical pathway that is important in regulating functions of the cardiovascular system as well as those of the central and peripheral nervous systems. PDE5 counterbalances the cGMP increase by hydrolyzing cGMP in a very efficient manner, resulting in an intracellular decrease in cGMP. Thus, PDE5 inhibition blocks cGMP hydrolysis and leads to an increase of intracellular cGMP and initiates the cGMP-driven cascade of downstream reactions. Ultimately these pathways lower intracellular calcium level and decrease sensitivity to calcium, thereby promoting relaxation of smooth muscle cells (SMC) and inhibition of a variety of other calcium-dependent processes. The substantial expression of PDE5 in SMC in the vascular system and in platelets directed the attention of pharmaceutical companies to the development of PDE5 inhibitors for treatment of cardiovascular diseases, e.g., hypertension and/or coronary heart disease. However, despite huge efforts these indications could not be successfully treated with PDE5 inhibitors and the projects were terminated. This failure may be due to the fact that the effect of PDE5 inhibitors to raise intracellular cGMP level requires ongoing cGMP formation. Strong evidence to support this concept is the highly efficacious treatment of ED with PDE5 inhibitors.

The mechanism of penile erection as well as the role of PDE5 inhibition have been extensively studied and reviewed (Anderson and Stief 1997; Carson and Lue

2005; Francis and Corbin 2005; Lue 2000). Penile erection is a result of the relaxation of arterial and other smooth muscle tissue within the penis. Relaxation of arterial smooth muscle is accompanied by increased blood flow to the penile corpora. The state of contractile tone in penile tissue is determined by a dynamic balance of contractile and relaxing mechanisms. The prominent role of NO as a neurotransmitter during sexual stimulation is the basis for the specificity of PDE5 inhibitors for the treatment of ED (Burnett 2002; Bush et al. 1992; Ignarro et al. 1990; Rajfer et al. 1992; Trigo-Rocha et al. 1994). During either direct or psychogenic sexual stimulation, NO is synthesized by neuronal NO synthase (nNOS) in the nerve terminals of parasympathetic, non-adrenergic, non-cholinergic (NANC) neurons in the penis and also by endothelial NO synthase (eNOS) in the endothelial cells of the blood vessels and the lacunar spaces of the corpora cavernosa. NO activates smooth muscle cell NO-sensitive guanylyl cyclase, resulting in increased intracellular cGMP level, which leads to relaxation of smooth muscle in the corpus cavernosum and in penile arterioles. The level of cGMP is regulated by its rate of synthesis via the NO-sensitive guanylyl cyclase and its hydrolysis to 5′-GMP by cGMP-hydrolyzing PDEs.

PDE5 is the most prominent cGMP-hydrolyzing PDE in the human corpus cavernosum, but some PDE2 activity has also been described, as well as significant amounts of the cAMP-metabolizing PDE3 and PDE4 (Boolell et al. 1996; Taher et al. 1997). Inhibition of PDE5 leads to an increase in the level of cGMP, thus enhancing the relaxation of SMC. Consequently, the vascular tone in penile arteries decreases. This causes increased blood flow and an enlargement of the cavernosal tissue, which induces penile erection (Nehra et al. 1999; Schultheiss and Stief 1999). NO-mediated vasorelaxation is the basis for the therapeutic application of PDE5 inhibitors in the treatment of ED. The release of NO by NANC nerves and/or by the endothelium is impaired under pathological conditions such as diabetes and/or coronary artery disease, hypertension, or spinal cord injury, which consequently leads to reduced cGMP synthesis (Kapur and Schwarz 2007). Through PDE5 inhibition, a sufficient level of cGMP leading to erection in many patients with ED can still be attained; this is clinically important in patients with diabetes, for example, where impaired NO release might be the cause of the ED. However, the clinical effectiveness of PDE5 inhibition depends on at least a minimal NO signal in order to induce some increase in cellular cGMP. Otherwise, PDE5 inhibitors are ineffective in inducing vasorelaxation. Complete destruction of the cavernosal nerves during radical prostatectomy or severe diabetic neuropathies could be pathological conditions that principally limit the success of treatment with PDE5 inhibitors (Tejada 2004).

Typical side effects of PDE5 inhibitors, e.g., flushing, stuffy nose and dyspepsia, reflect the pharmacodynamic effects of PDE5 inhibition based on its expression profile in different tissues. Clinical efficacy of the available PDE5 inhibitors has been proven in many clinical studies worldwide and will not be reviewed here (Burnett 2006; Carson and Lue 2005; Dangrell 2005; Uckert et al. 2003). Also, development of newer PDE5 inhibitors for treatment of ED and combinations of therapies were recently reviewed intensively (Sandner et al. 2007).

3.1.3 New Treatment Options with PDE5 Inhibitors

PDE5 inhibitors are the gold standard for treatment of ED. Their efficacy, wide availability, safety and high tolerability have made them the focus of extensive research by both basic researchers and clinicians in recent years. PDE5 inhibitors may be optimally suited for the treatment of cardiovascular diseases due to their mechanism of action (Ravipati et al. 2007). Increases in intracellular cGMP level cause relaxation of vascular smooth muscles. This is not a generalized vasodilation, since its extent depends on the physiological stimulus NO. In regions where a relatively large NO production signals a high need for local perfusion, the chosen PDE5 inhibitor should be more efficacious than in regions with lower perfusion demand. In contrast to unspecific vasodilators, PDE5 inhibitors trigger a demand-driven increase in flow which may offer the opportunity for a very specific redistribution of blood flow into areas with the highest perfusion needs. Many of the new indications for PDE5 inhibitors (Table 2) are characterized by regional deficiencies in blood supply which can be modulated via the NO-cGMP pathway. The use of PDE5 inhibitors may be beneficial due to their capacity to reverse endothelial dysfunction and to selectively improve NO-modulated regional blood flow in regions of greatest need.

Table 2 Potency, therapeutic potential and development status of PDE5 inhibitors

Compound	IC_{50} PDE5 (nM)	Therapeutic use	Development status	Originator
Sildenafil	3.5–10	ED PAH BPS	Launched 1998 Launched 2005 Phase II	Pfizer
Vardenafil	0.14–1	ED BPS	Launched 2003 Phase II	Bayer, Schering, Pharma
Tadalafil	1.8–10	ED PAH BPS	Launched 2003 Phase III Phase II	Lilly and Company
SLX 2104	0.04	Raynaud's disease Hypertension	Phase II Phase II	Surface Logix
Udenafil	8.2	ED	Launched 2005 in Korea	Dong-A Pharmaceutical
Mirodenafil	0.33	ED	Launched 2007, in Korea	SK Chemical
Dasantafil	[a]	ED	Phase II since 2004	Schering Corp.
Avanafil	5.2	ED	Phase II	Vivus Ltd.
UK-369003	[a]	ED, PAH, Urinary Tract Disease	Phase II	Pfizer

BPS benign prostate syndrome; *PAH* pulmonary arterial hypertension; *ED* erectile dysfunction;
[a] not reported.

Pulmonary Hypertension

The most prominent example and currently the most investigated and established cardiovascular use of PDE5 inhibitors other than for treament of ED is PAH. The treatment of PAH with PDE5 inhibitors reduces vascular resistance in the pulmonary circulation and pressure in the pulmonary arteries. The rationale for these effects is based on the hypothesis that oxygenation of the alveoli stimulates release of NO, which causes a local relaxation of pulmonary resistance vessels. Inhibition of cGMP breakdown results in amplification of this regulatory pathway, causing increased vasodilation without inducing a ventilation-perfusion mismatch. (Ghofrani et al. 2004).

Even by 1996 Cohen et al. had already shown that a new selective and potent PDE5 inhibitor E4021 was as effective as inhaled NO in a rat model. Long-term treatment with oral E4021 caused selective pulmonary vasodilation and attenuated the increase in right ventricular hypertrophy and pulmonary arterial remodelling induced by chronic hypoxia in rats (Takahashi et al. 1996). Furthermore, it has been demonstrated in several animal models that sildenafil reduces pulmonary arterial pressure and right heart hypertrophy as well as hypoxia-induced rise in pulmonary pressure. Moreover, sildenafil partially reverses pulmonary artery muscularization (Schermuly et al. 2004; Sebkhi et al. 2003). Sildenafil has been shown in many clinical studies to be a safe and effective drug for patients with PAH (Galie et al. 2005; Ghofrani et al. 2002; Sastry et al. 2004). Sildenafil (Revatio®) was approved in 2005 for the treatment of patients with PAH. This successful development for PAH demonstrates that it is possible to take advantage of both hemodynamic and anti-remodelling mechanisms mediated by PDE5 inhibition (Guazzi and Samaja 2007).

Treatment Options Based on Cardiac Effects of PDE5 Inhibitors

Although the level of PDE5 in cardiomyocytes is rather low (Loughney et al. 1998; Stacey et al. 1998) it is becoming increasingly evident that PDE5 together with PDE1 and PDE2 plays an important role in the regulation of cGMP of cardiomyocytes (Kass et al. 2007). This regulation seems to be compartmentalized so that PDE5 controls the NO-sGC generated cGMP pool (Castro et al. 2006; Takimoto et al. 2007). PDE5 inhibition has little effect on basal contractility but suppresses acute beta-adrenerigic stimulation in dog (Senzaki et al. 2001), mouse (Takimoto et al. 2005a), and human heart (Borlaug et al. 2005). In a mouse model, PDE5 inhibition has also been shown to reverse pressure overload-induced ventricular hypertrophy which mimics congestive heart failure (CHF) (Takimoto et al. 2005b). Moreover, PDE5 inhibitors reduce post-ischemic dysfunction (Fisher et al. 2005) as well as attenuate apoptosis and necrosis in isolated cardiomyocytes (Das et al. 2005). In a model of isoproterenol-induced cardiac hypertrophy in rats, survival was improved by daily dosing of sildenafil; in addition, a negative correlation was observed with cardiac hypertrophy and troponin-T release (Hassan and Ketat 2005). Potential mechanisms by which PDE5 inhibitors could exert positive effects on

CHF may affect not only cardiomyocytes but also SMC of cardiac and or peripheral blood vessels. To date at least some beneficial effects on cardiac function have been demonstrated in small clinical studies. The most prominent mechanism is a cGMP-mediated relaxation of SMC which elicits a direct vasodilation. The positive effect of direct vascular dilation may be important in large blood vessels, since an improved compliance of the large arteries reduces peak ventricular pressure and improves peripheral perfusion during diastole by lowering pulse pressure. Such an effect has been demonstrated (Hirata et al. 2005).

Another potential benefit of PDE5 inhibitors may be an improvement of endothelial function. A placebo-controlled study showed that acute PDE5 inhibition with sildenafil increases endothelium-dependent, flow-mediated vasodilation in patients with chronic heart failure (Hryniewicz et al. 2005; Katz et al. 2000). More recently, studies show that PDE5 inhibitors increase circulating endothelial progenitor cells (Foresta et al. 2005). Given that a higher number of circulating progenitor cells is directly linked with a lower incidence of CV events, this might be another site of action for PDE5 inhibitors on cardiovascular diseases. In summary, there is substantial evidence that PDE5 inhibitors may have positive effects in the treatment of cardiovascular disease. However, the potential mechanisms of action are not fully understood and the clinical benefit remains to be demonstrated.

Raynaud's Disease

There is increasing evidence that the NO/cGMP system plays a major role in the pathophysiology of Raynaud's disease (Freedman et al. 1999). Raynaud's disease is characterized by painful temperature-sensitive, digital vasospasms leading to pale, cyanotic skin mostly limited to the digits (Pope 2007). In several clinical studies, sildenafil and vardenafil have been shown to improve peripheral blood flow, decrease the severity of clinical symptoms, and reduce the frequency and cumulative attack duration in some Raynaud's patients (Caglayan et al. 2006; Fries et al. 2005).

Female Sexual Dysfunction

Several attempts have been made to use PDE5 inhibitors for the treatment of female sexual dysfunction (FSD). There is some preclinical evidence that PDE5 plays a functional role in vaginal and clitoral smooth muscles. (Oelke et al. 2006; Park et al. 1998; Uckert et al. 2005). This raises the expectation that PDE5 inhibitors may improve vaginal and clitoral blood flow and facilitate arousal and orgasm in women. However, to date no convincing set of clinical studies is available supporting use of PDE5 inhibitors in FSD.

Urological Indications

Clear evidence of the presence of PDE5 in various urogenital tissues such as human prostate, human bladder and urethra (Tinel et al. 2006; Uckert et al. 2001, 2006)

raised the expectation that PDE5 inhibitors could be beneficial for the treatment of benign prostate syndrome (BPS). BPS comprises lower urinary tract symptoms (LUTS) and benign prostate hyperplasia (BPH), which can cause bladder outlet obstruction. There is recent evidence that the PDE5 inhibitors vardenafil, sildenafil and tadalafil can relax prostate, bladder and urethral tissues in vitro (Tinel et al. 2006). Moreover, preclinical research has demonstrated a reduction of non-voiding contractions in vivo in rats treated with vardenafil or sildenafil (Filippi et al. 2006; Tinel et al. 2006). Such data are the basis for several encouraging phase II clinical studies using sildenafil, vardenafil and tadalafil in men suffering from symptomatic BPH where a reduction of LUTS was reported (McVary et al. 2007a, b; Stief et al. 2008).

There is some preclinical evidence that PDE5 inhibitors may be useful in the treatment of Peyronie's disease (PD), a disorder that is characterized by development of fibrotic plaques of the tunica albuginea of the penis; the proliferation and accumulation of fibroblasts in the penis promote PD so one possible therapeutic approach is the prevention of fibroblast proliferation. It has been demonstrated that the NOS/NO/cGMP system plays an important role in the progression of plaque development since inhibition of inducible NO synthase (iNOS) results in exacerbated fibrosis (Ferrini et al. 2002). In primary cultures of human PD-derived fibroblasts, sildenafil reduced collagen I synthesis and myofibroblast differentiation as well as increasing apoptosis in vitro (Valente et al. 2003). Very recently, studies using an in vivo rat model of PD demonstrated that vardenafil significantly decreased collagen I and III deposition and reduced the numbers of myofibroblasts in PD plaques (Ferrini et al. 2006).

Effects of PDE5 Inhibitors in CNS

Evidence to support the potential use of PDE5 inhibitors for the treatment of CNS disorders is mainly based on the presence of PDE5 in morphologically distinguishable structures in the CNS (Blokland et al. 2006; Van Staveren et al. 2003). Together with an increasing number of reports on the effects of PDE5 inhibitors on the CNS (Blokland et al. 2006; Menniti et al. 2006), this suggests that the NO/cGMP pathway may be involved in a multitude of cerebral functions and may play a central role in brain metabolism. Several in vitro and in vivo studies have investigated the effects of selective PDE5 inhibitors, indicating that PDE5 inhibitors have the potential to improve memory performance in animals, particularly in the early consolidation process of long-term memory (Baratti and Boccia 1999; Devan et al. 2006; Prickaerts et al. 2002, 2004). Taken together, the encouraging effects of sildenafil in several rat models of stroke (Zhang et al. 2005a, 2006) and the positive effects on enhancement of recognition raise some optimism for future treatment options, although further evaluation is required.

3.2 Inhibitors of PDE6

To date, there are no inhibitors that are selective for the PDE6 family, and with the exception of tadalafil (Cialis), most inhibitors described as "PDE5-selective" have similar potency for inhibition of PDE5 and the PDE6 isoenzymes (Zhang et al. 2005b); tadalafil potency is more than 200-fold greater for PDE5 than for either PDE6 isoform. Other inhibitors marketed as "PDE5-selective" including dipyridamole, T-1032, T-0156, zaprinast, sildenafil, E4021, and vardenafil are not selective between PDE5 and PDE6 (Zhang et al. 2005b). Again, this emphasizes the problems with labeling inhibitors with the term "selective." Zaprinast is the most selective inhibitor since it is ~10-fold more potent for both the cone and rod isoforms of PDE6 ($IC_{50} = 32$ and 30 nM, respectively) than for PDE5. Vardenafil is the most potent inhibitor for both the cone and rod enzymes (0.3 and 0.7 nM), and this potency approaches that for PDE5. The potency of vardenafil, the selectivity of zaprinast, and low affinity of tadalafil for PDE6 could be important leads in efforts to develop inhibitors that target PDE6.

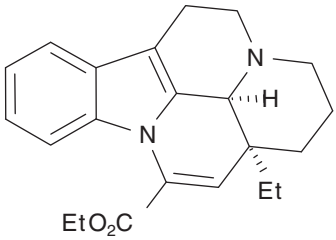
3.3 Inhibitors of PDE9

A potent PDE9-selective inhibitor (BAY 73–6691) that inhibits recombinant human PDE9A with an $IC_{50} = 55$ nM has recently been reported (Wunder et al. 2005). BAY 73–6691 also inhibits PDE1C and PDE11A, albeit weakly ($IC_{50} = 1400$ and 2600 nM, respectively). The IC_{50} of this compound for inhibition of other PDEs (PDEs 2A, 3B, 4B, 7B, 8A, and 10A) exceeds 4000 nM. Vardenafil and SCH 51866 also inhibit PDE9 ($IC_{50} = 580$ and 1600 nM, respectively), but these compounds are significantly more potent for PDE5 ($IC_{50} \sim 0.1$–0.4 nM and 100 nM, respectively); SCH 51866 also inhibits PDE1 with an $IC_{50} \sim 100$ nM (Kotera and Omori 2006; Soderling et al. 1998; Vemulapalli et al. 1996).

4 PDE Inhibitors of PDEs with Dual Specificity

4.1 PDE1 Inhibitors

PDE1 modulation has not been firmly tied pharmacologically to the treatment of any specific disease although it has been speculated as having an important role for neurodegenerative diseases (Menniti et al. 2006) as well as for heart failure (Vandeput et al. 2007). In contrast to PDE3, PDE4, and PDE5, which have received considerable attention from medicinal chemists, there is much less information available on inhibitors of PDE1. For many years the alkaloid vinpocetine (Fig. 6) could be cited as the most significant selective, albeit weak, PDE1 inhibitor, but vinpocetine



Vinpocetine
IC_{50}: 20 μM

Fig. 6 Example of a selective PDE1 inhibitor

also inhibits PDE6 isoforms with potency similar to that for PDE1 (Zhang et al. 2005b). More recently two selective and potent PDE1 inhibitors from ICOS Corporation (structures not disclosed) were used as tools to demonstrate a role of PDE1 in human cardiac myocytes IC229 IC50 = 560 nM (Vandeput et al. 2007) and in regulation of lipolysis in human adipocytes (IC224 IC50 = 800 nM) (Snyder et al. 2005). Such compounds could help to identify which of the above mentioned physiological processes could be a therapeutic relevant target.

4.2 PDE2 Inhibitors

PDE2 is thought to be involved in regulating many different intracellular processes, including aldosterone secretion from the adrenal gland, intracellular concentrations of cAMP and cGMP in platelets, cGMP level in neurons (Hepp et al. 2007), effect on long-term memory (Boess et al. 2004), modulation of cardiac L-type Ca^{++} current (Fischmeister et al. 2005), and barrier function of endothelial cells under inflammatory conditions (Seybold et al. 2005).

The biochemical and pharmacological characterization of PDE2 would be clearly advanced by the availability of potent, selective inhibitors. For many years, the only PDE2 inhibitor available has been EHNA, but its use is complicated by its action as a potent adenosine deaminase inhibitor (Fig. 7); the much lower affinity of EHNA for PDE2 inhibition ($IC_{50} = 800$ nM) was not discovered until 1992 (Podzuweit et al. 1995). EHNA is selective for PDE2 over other PDEs, but its dual pharmacological activity presents a problem for use as a biochemical tool. More recently, Bayer has disclosed several potent and selective PDE2 inhibitors BAY 60–7550 (Fig. 7) ($IC_{50} = 4.7$ nM) (Boess et al. 2004; Hendrix and Kallus 2004) and PDP (9–6-**P**henyl-2-oxohex-3-yl)-2-(3,4-**d**imethoxybenzyl)-**p**urine-6-one) ($IC_{50} = 0.6$ nM) (Seybold et al. 2005). PDE2 has a broad spectrum of regulatory functions and it should be a future goal to define pathological conditions where a PDE2 inhibitor could exert beneficial effects.

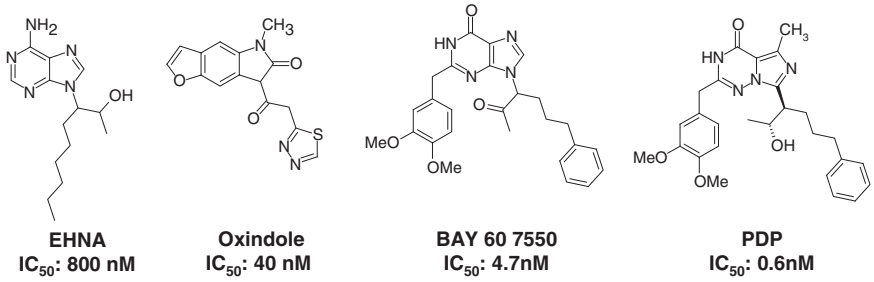


Fig. 7 Selective PDE2 inhibitors. * PDP: (9–6-**P**henyl-2-oxohex-3-yl)-2-(3,4-**d**imethoxybenzyl)-**p**urine-6-one)

4.3 PDE3 Inhibitors

Two gene products, PDE3A and PDE3B, with very similar kinetic properties have been found. A membrane-anchoring domain is a special feature of the particulate PDE3s that are found in adipocytes and hepatocytes. Both PDE3A and PDE3B activity are regulated by phosphorylation-induced (PKA and PI3K/PKB) hormonal stimulation; this occurs in platelets and heart, e.g., via the action of prostaglandins and epinephrine, and also via the action of leptin, glucagon and insulin. PDE3A is highly expressed in platelets, vascular smooth muscle, and cardiac myocytes, whereas PDE3B is predominant in adipose tissue, liver and pancreas but also in some cardiovascular tissues (Degerman et al. 1997; Shakur et al. 2001). PDE3 inhibitors block platelet aggregation, increase myocardial contractility, induce vascular smooth muscle relaxation and block oocyte maturation.

Research on the physiological role of PDE3 was clearly facilitated by the availability of a variety of PDE3-selective inhibitors (Fig. 8) (Hendrix and Kallus 2004). This formed the basis for the use of PDE3 inhibitors such as milrinone ($IC_{50} = 2.4\,\mu M$ (Primacor®) in the treatment of heart failure. Based on positive inotropic effects, peripheral vasodilation, which is induced by vascular smooth muscle relaxation, and platelet inhibitory activity, these PDE3 inhibitors exhibited a favorable and beneficial clinical profile in treatment of cardiac heart failure. Unfortunately, during chronic use, treatment has been associated with an increase in mortality due to a higher frequency of lethal arrhythmias. To date, only an i.v. formulation is used in treatment of acute heart failure. Another PDE3 inhibitor, cilostazol (Pletal®), $IC_{50} = 0.2\,\mu M$, is approved in the United States and some European countries for the treatment of intermittent claudication. NT-702 with $IC_{50} = 0.18\,nM$, is in clinical development (Ishiwata et al. 2007).

A crystal structure of the PDE3B C domain together with the inhibitor cilostamide has been published by (Scapin et al. 2004) and will facilitate the design of new inhibitors. To date, no PDE3 inhibitor has been synthesized which clearly discriminates between PDE3A and PDE3B. A PDE3B-selective inhibitor could be of interest for influencing PDE3 action in insulin- and leptin-signaling pathways (Shakur et al. 2001; Zhao et al. 1998, 2002, 1997b). Because of the

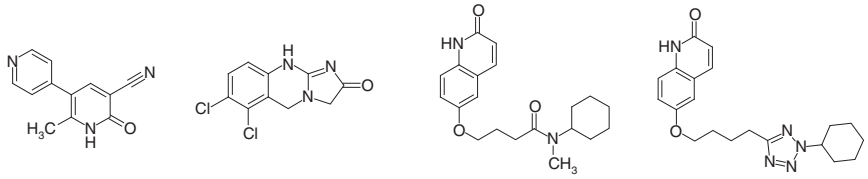


Fig. 8 Examples of selective PDE3 inhibitors

supposed effects of leptin on food intake and body weight, the possible role of this enzyme in disorders like diabetes and obesity suggests attractive targets for a PDE3A/B selective inhibitor.

4.4 PDE10 Inhibitors

PDE10 hydrolyzes both cAMP and cGMP. Due to higher affinity, cAMP inhibits cGMP hydrolysis. Interest in this more recently discovered PDE (Soderling et al. 1999) has been stimulated by its high-level expression in the striatal region of the brain. It is also expressed in cerebellum, thalamus, hippocampus, spinal cord and testes, although a wide variability among different species has been seen (Coskran et al. 2006; Seeger et al. 2003). PDE10 knockout mice have impaired exploratory activity and delayed acquisition-avoidance behavior (Siuciak et al. 2006). Little is known about PDE10 regulation either in vitro or in vivo.

To date, no selective PDE10 inhibitor has been disclosed. Papaverine, a classical PDE inhibitor that was previously thought to be nonspecific, is a reasonably selective and potent inhibitor (18 nM) of this enzyme (Fig. 9). The next closest IC_{50} values are those for PDE4D and 4C (320 and 808 nM, respectively), and for PDE6 (860 nM) (Lebel et al. 2003). Papaverine was shown to improve cognitive deficits in a rat model (Rodefer et al. 2005). Apart from this isolated example, Bayer has disclosed a number of PDE10 inhibitors falling into two different chemical series (Erguden et al. 2003; Hendrix and Kallus 2004; Niewoehner et al. 2003). It is suggested that PDE10 might be a target for treatment of psychiatric disorders (Rodefer et al. 2005; Siuciak et al. 2006). More potent and selective inhibitors will probably be necessary to clarify the potential of PDE10 as a drug target.

4.5 PDE11 Inhibitors

PDE11 is the most recently identified PDE family (Fawcett et al. 2000). In humans, PDE11 expression is strong in prostate and moderate in testis and other tissues

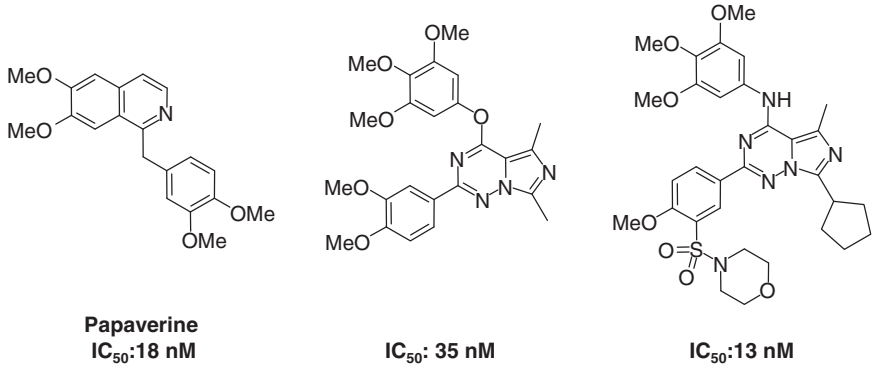


Fig. 9 PDE10 inhibitors. The two structures in the right portion of the figure are taken from patents and have no official names (Erguden et al. 2003; Hendrix and Kallus 2004; Niewoehner et al. 2003)

such as skeletal muscle. However, many differences among species have been seen (Loughney et al. 2005; Yuasa et al. 2000). Subcellular localization is unknown.

The physiological role of PDE11 is unknown. PDE11 knockout mice (Wayman et al. 2005) demonstrate mildly impaired sperm function and spermatogenesis but the mice are fertile. The most potent inhibitor for PDE11, tadalafil, was found unintentionally during its clinical development. Inhibition for PDE11 isoenzymes differs by as much as fivefold. IC_{50} for PDE11A4 and PDE11A1 of 73 and 15 nM, respectively is 5–40-fold weaker compared to PDE5 IC_{50} (Weeks et al. 2005, 2007). Zaprinast and IBMX also inhibit PDE11, albeit with IC_{50} in the μM range. New highly selective and potent inhibitors clearly could help to clarify the physiological role of PDE11.

5 Concluding Remarks

The catalytic functions and regulatory mechanisms associated with actions of PDEs in cellular signaling processes have evolved for precise regulation of cN levels. Common features are shared among different PDE families, but even slight differences in structure, tissue abundance, sub-cellular localization, and other features contribute importantly to providing a refined and highly controlled regulation of the action of these proteins and their impact on cN signaling in all tissues. These same features frequently impact potency and selectivity of PDE inhibitors and their modes of action in tissues. In some cases, effects of certain signaling agents or inhibitors are mediated by modulation of a particular PDE, whereas in other instances the effects reflect a composite of PDE action in that tissue. A general concept is also emerging that PDEs provide for negative feedback regulation of cAMP and cGMP pathways. Since practically all extant PDE inhibitors act at the PDE catalytic sites, they block negative feedback in addition to elevating the CN level.

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cGMP-Dependent Protein Kinase Modulators

Elke Butt

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Abstract The first cGMP-dependent protein kinase (PKG) modulators were described nearly 30 years ago and since then more than 200 compounds have been synthesized and tested, but only a small subset of these compounds has found widespread application. The aim of this review is to suggest a framework for evaluating and using PKG activators and inhibitors and to explore and interpret PKG signal transduction in cell culture-based model systems. Therefore, cross-reactivity of cGMP-analogs with other classes of cyclic nucleotide binding proteins, as well as the advantages and problems of newly designed PKG inhibitors, are discussed.

Additional information and a search option are available at www.cyclic-nucleotides.org.

Keywords: PKA · PKG Iα · PKG Iβ · PKG II · cGMP · Rp-cGMPS · 8-Br-cGMP · 8-pCPT-cGMP · 8-Br-PET-GMP · 8-APT-cGMP · DT-2 · DT-3 · KT5823 · H89 · caged-cGMP · Phosphodiesterases

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1 Introduction

Six years after the discovery of cAMP by Earl Sutherland in 1958 (Sutherland and Rall 1958), cGMP was isolated from rat urine (Ashman et al. 1963). The cGMP-dependent protein kinase (PKG), originally discovered by Kuo and Greengard in 1970 from lobster tail muscle, was first purified and characterized from bovine lung (Gill et al. 1976). Subsequently, the complete mammalian amino acid sequence was elucidated (Takio et al. 1984). The soluble PKG type I exists in two isoforms (type Iα and Iβ) generated by separate promoters from the same gene (Orstavik et al. 1997). A distinct, primarily membrane-bound form (PKG type II) was originally identified in and cloned from epithelial cells of the small intestine (De Jonge 1981; Jarchau et al. 1984). All the three proteins are composed of an amino-terminus, two cGMP binding sites with different affinities and a catalytic domain. Due to a hydrophobic leucine zipper in the N-terminus, all three PKGs are homodimers (for more structural and biochemical properties see: Pfeifer et al. 1999; Lohmann and Walter 2005).

Although the cGMP binding and catalytic domains of the soluble α and β forms are identical, the kinetic parameters differ. Analysis of recombinant chimeric PKG Iα/Iβ kinases identified the amino-termini being important for the different binding constants and cooperativity between the two cGMP binding sites in the protein kinases (Ruth et al. 1997) resulting in an apparent K_a of 0.1 μM for PKG Iα and of 1 μM for PKG Iβ (PKG II: 0.07 μM, see also Table 1).

Cyclic nucleotide analogs and other small molecules have emerged as indispensable tools for studying PKG signal transduction. Apart from PKG there are other cGMP-binding proteins like cyclic nucleotide regulated channels (CNGs) (Craven

Table 1 Apparent activation (K_a) and dissociation (K_d) constants. The cyclic nucleotide activation constants (K_a) for PKA I and II holoenzymes were determined in vitro with biotinylated Kemptide as substrate. The activation constants for the purified PKG kinases Iα, Iβ and II were measured by the phosphocellulose method using the substrate VASPtide. For determination of Epac dissociation constants (K_d) efficiency of cGMP-analogs to compete with [^{3}H]-labelled cAMP for binding to Epac-1 was measured. No Epac activation constants are given since at a cyclic nucleotide concentration of 500 μM the induced activity remained less than 10% of the k_{max} for cAMP

Activators	PKG Iα K_a (μM)	PKG Iβ K_a (μM)	PKG II K_a(μM)	PKA I K_a(μM)	PKA II K_a(μM)	Epac K_d(μM)	CNG K_a(μM)
cGMP	0.1[a]	0.9[a]	0.04[a]	9[b]	60[c]	40[d]	1.6[e] (olf) 1500[f] (rod)
8-Br-cGMP	0.01[a]	1.0[a]	0.025[a]	30[b]	12[c]	25[d]	6.7[g] (rod)
8-pCPT-cGMP	0.04[a]	0.9[a]	0.004[a]	4[b]	7[c]	0.9[d]	0.07[h] (olf) 0.5[g] (rod)
8-Br-PET-cGMP	0.013[i]	0.009[i]	0.02[d]	18[b]	30[b]	12[b]	
8-APT-cGMP	0.01[d]	3.1[d]	0.06[d]		20[d]		

[a] Pöhler et al. 1995; [b] E. Butt unpublished results; [c] Butt et al. 1992; [d] Poppe et al. 2008; [e] Ludwig et al. 1990; [f] Tanaka et al. 1989; [g] Wei et al. 1998; [h] Strassmaier and Karpen 2007; [i] Sekhar et al. 1992

and Zagotta 2006) and phosphodiesterases (PDEs) (Bender and Beavo 2006) that might be affected by these widely used compounds. Here I summarize published data and propose guidelines to help interpreting observations on PKG signal transduction in cell models. Nevertheless, the readers should always keep in mind that the constants summarized in this review were obtained by in vitro assays with purified enzymes and do not necessarily reflect the situation in a living cell.

1.1 Cyclic Nucleotide Analogs

During the last 30 years, many analogs of cAMP and cGMP were developed, particulary in the field of cAMP-dependent protein kinase (PKA) and PKG cyclic nucleotide based activators and inhibitors (Schwede et al. 2000a; Bain et al. 2003), to meet the following requirements: (1) Membrane permeability: since cAMP and cGMP are unable to penetrate intact cell membranes, several structural modifications were introduced, leading to increased lipophilicity and consequently improved membrane permeability. The so-called lipophilicity index, as can be determined by high performance liquid chromatography analysis (Krass et al. 1997), is a useful method to compare membrane permeability of cyclic nucleotides, (2) Specificity towards defined cyclic nucleotide receptors: especially the discovery of exchange proteins directly activated by cAMP (Epac) raised the issue whether cGMP analogs might cross-activate PKA and Epac signalling, (3) Resistance to hydrolysis by PDEs: PDEs can reduce the half-life of cGMP analogs in vivo drastically and thus improved resistance to hydrolysis was considered to be beneficial.

The chemical structures, IUPAC names and abbreviations of the cGMP-analogs discussed in this review are shown in Fig. 1.

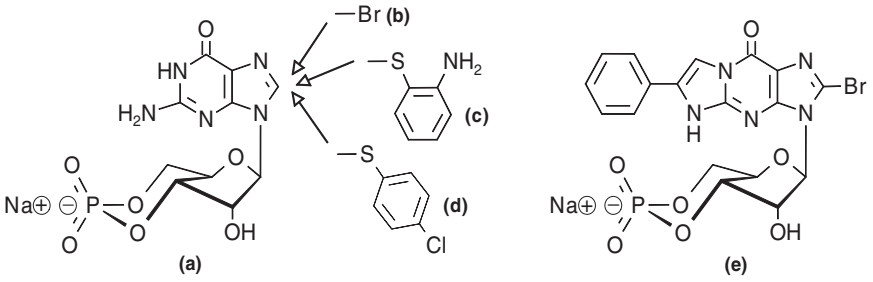

Fig. 1 Chemical structures, and names of cGMP analogs a cGMP, Guanosine-3′, 5′-cyclic monophosphate; **b** 8-Br-cGMP, 8-Bromoguanosine-3′, 5′-cyclic monophosphate; **c** 8-APT-cGMP, 8-(4-Aminophenylthio)guanosine-3′, 5′-cyclic monophosphate; **d** 8-pCPT-cGMP, 8-(4-Chlorophenylthio)guanosine-3′, 5′-cyclic monophosphate; **e** 8-Br-PET-cGMP, ß-Phenyl-1, N^2-etheno-8-bromoguanosine-3′, 5′-cyclic monophosphate

1.1.1 Activators

A variety of cGMP analogs are described in the literature and are commercially available. Recently, these analogs were systematically tested for their ability to activate PKG Iα, PKG Iβ and PKG II, for cross-reactivity with PKA or Epac and for their hydrolysis by PDEs (Poppe et al. 2008) (Tables 1 and 2).

The most potent activator of PKG is 8-Br-PET-cGMP, which shows very high affinities for both PKG type I and type II. 8-Br-PET-cGMP is stable against degradation by all tested PDEs but inhibits PDE2, PDE4 and PDE6 with a K_i that is in the same order of magnitude as the K_m for their native substrate.

8-APT-cGMP is suited to discriminate between the isoforms of PKG I, since it activates PKG Iα with a K_a of 0.01 μM and PKG Iβ at 3.1 μM (PKG II: 0.06 μM; PKA II: 20 μM).

PKG II is best activated by 8-pCPT-cGMP but the compound is hydrolysed by PDE5 and PDE10, and surprisingly, to some extent by the cAMP-specific PDE4. It should be noted that 8-pCPT-cGMP is also binding to Epac with a K_d of 0.9 μM (K_d for cAMP: 2.9 μM) but without any activation (Table 1). Therefore this compound might interfere with Epac-mediated effects by competing with cAMP for the binding site.

1.1.2 Inhibitors

The Rp-diastereomers of cGMP are competitive inhibitors at the cyclic nucleotide binding site of the PKGs. With a sulphur in the equatorial position at the phosphate moiety (Fig. 1), these compounds bind to PKG but apparently do not evoke the conformational change of the enzyme required for activation (Dostmann 1995; Zhao et al. 1997). In addition, the equatorial sulphur sterically hinders the opening of the cyclic phosphate ring by phosphodiesterases and hence, reduces hydrolysis more than 10,000-fold (Van Haastert et al. 1983).

Attention should be paid while storing Rp-analogs for longer periods (>2 days) in solution as the antagonists are slowly metabolized to agonists by oxidation process. For example, when using 100 μM Rp-8-pCPT-cGMPS an oxidation of only 0.01% will generate 0.01 μM of the 8pCPT-cGMP–sufficient to activate PKG. Therefore, anti-oxidizing assay conditions and freeze-dried storage of the Rp-compounds are highly recommended.

Concerning the inhibitory phosphorothioate cGMP analogs, Rp-cGMPS is only a weak and non-specific antagonist since it inhibits both PKG and PKA with similar apparent inhibition constants of 20 μM. Rp-8-Br-PET-cGMPS was found to be the most potent inhibitor for PKG I (K_i of 0.03 μM) with a more than 300-fold selectivity over PKA II (K_i of 11 μM) while inhibition of PKG II occured at 0.9 μM. In rat tail arteries, human platelets (Butt et al. 1995) and intestinal mucosa (Vaandrager et al. 1997), the Rp-derivative has been tested successfully to selectively inhibit PKG mediated effects. Both Rp-cGMPS analogs investigated are inhibitors of all tested phosphodiesterases (Table 2). With PDEs 2, 5, 6 and 10, carrying an allosteric GAF

Table 2 Hydrolysis of cGMP-analogs by phosphodiesterases. The kinetic data for the cGMP-analogs with purified full-length PDEs were measured in vitro using isothermal microcalorimetry. K_m and K_i values are given in μM and V_{max} values are μmoles/min/mg protein

Activators and Inhibitors	PDE 1A			PDE 1B			PDE 1C			PDE 2			PDE4			PDE 5			PDE 6			PDE 10		
	K_m	V_{max}	K_i	K_m	V_{max}	K_i	K_m	V_{max}	K_i	K_m	V_{max}	K_i	K_m	V_{max}	K_i	K_m	V_{max}	K_i	K_m	V_{max}	K_i	K_m	V_{max}	K_i
cGMP	8.2	20		5.3	2.6		4.6	13.7		31	176				25	2	7		10	410		1.1	1.56	
8-Br-cGMP			47			4.4			63			91			30			79			33	23	0.5	
8-pCPT-cGMP		no effect				8.6			47			40	53	2		27	0.75				41	6	3.6	
8-Br-PET-cGMP			2.4			2.1	73	3.5		98	175				9			5.3			11			34
Rp-8-pCPT-cGMPS			42.4			2.1			33		no effect				137			50			26			38
Rp-8-Br-PET-cGMPS		no effect				2.5			56			0.76			8			4			14			5

all data: Poppe et al. (2008)

domain, a linear mixed type of inhibition is observed, assuming a binding of the Rp-derivative to this cGMP-binding site and preventing the hydrolysis of cGMP and cAMP.

The ability of an analog to be hydrolyzed by a PDE would be expected to decrease the in vivo/in situ half-life of the analog and thereby lower the potency of the compound, particularly at long incubation times. Similarly, for the analogs that can act as competitive inhibitors of a PDE, there is substantial potential for the compound to alter endogenous cAMP or cGMP concentration. For example, in cells expressing PDE1B (e.g. neurons, macrophages, lymphocytes) cyclic nucleotide analog data should be interpretated very cautiously, as all tested cGMP-derivatives inhibit PDE1B at low concentrations, and thus may lead to elevated cAMP and/or cGMP concentrations.

For completeness it should be mentioned that Rp-analogs act as activators on CNG channels, although at high concentrations ($>100\,\mu M$) the compounds are non-competitive inhibitors (Wei et al. 1998).

1.1.3 Caged Cyclic Nucleotides

"Caged" compounds are inactive, photolabile derivatives of a biological active compound. After a UV flash the light sensitive group is released and the active molecule is liberated (Adams and Tsien 1993). Caged-cGMP (4,5-dimethoxy-2-nitrobenzyl-cGMP) is uncharged at physiological pH and can readily cross biological membranes. Another light-sensitive cGMP is the recently developed coumarin-cGMP analog (BCMCM-caged-cGMP). For this new compound only little experience and data are available (Hagen et al. 2003). However, both compounds release cGMP that is not stable against degradation by phosphodiesterases (Table 2). The synthesis of caged 8-Br-cGMP provides a more hydrolysis-resistant analog that was successfully used in studies with cyclic nucleotide gated channels (Hagen et al. 1996).

Due to its polar ionic structure cGMP itself is not able to penetrate intact cellular membranes. Acetoxymethyl (AM)-ester has been successfully introduced to mask the charged cyclic phosphate, thus increasing lipophilicity and membrane permeability. Inside the cell the AM-ester is hydrolysed by esterases into acetic acid, formaldehyde and cyclic nucleotide. A concentration of $1\,\mu M$ cGMP-AM was reported to be as effective as $100\,\mu M$ 8-Br-cGMP for induction of long-term potentiation in rat hippocampal neurons (Zhuo et al. 1994). To avoid the fast metabolic turnover of cGMP, 8-Br-cGMP-AM and 8-pCPT-cGMP-AM have been synthesized and used as well (Schwede et al. 2000b). However, the newest data on cGMP-analog hydrolysis showed a degradation of 8-pCPT-cGMP with PDE5 [present in all tissues and cell types, with heart and cardiomyocites being contentious (Lin et al. 2006)] and a rapid hydrolysis of both, 8-Br-cAMP and 8pCPT-cGMP, with PDE10 (various tissues including cerebellum, putamen, thalamus, heart, testis and placenta [Fujishige et al. 1999)] (Table 2).

$N_2, O_{2'}{}^{-}$Dibutyryl-cGMP itself is inactive towards PKG as the $2'$-hydroxy-group at the ribose is blocked. Bioactivation of this compound occurs after diffusion into

the cell through intracellular hydrolysis of the 2′-butyrate by non-specific endogenous esterases or amidases, leading to N^6-monobutyryl cGMP, a poor activator of PKG Iα with a K_a of 1.8 μM (Corbin et al. 1986). However, the released butyrate itself generates several side effects that often interfere with second messenger pathways and was found to induce differentiation and growth inhibition in cancer cells (Cho-Chung et al. 1995; Kawamoto et al. 1998). Therefore, $N_2, O_{2'}$-Dibutyryl-cGMP is not an ideal compound for PKG signal transduction research and additional control experiments with sodium butyrate are highly recommended.

1.1.4 Cell Membrane Permeability

The negative charge at the phosphate is the major reason for the poor membrane permeability of cGMP. Introduction of hydrophobic substituents, bound mainly at the guanine moiety of cGMP (e.g. 8-pCPT-cGMP), improve membrane permeability, often accompanied by increased binding affinity. Basal cGMP levels were determined to be $\sim 10^{-8}$ M in most tissues (for cAMP the concentrations are 10x higher). As the in vitro activation constant K_a for PKG Iα is around 0.1 μM, full activation requires intracellular cGMP concentrations in the range of 0.5–1 μM. However, studies on cyclic nucleotide cell membrane permeability in C6 glioma cells with 8-Br-cAMP and 8-pCPT-cAMP revealed that only 10% of the analogs applied extracellularly could be detected inside the cell (Bartsch et al. 2003). The data are transferable to 8-Br-cGMP and 8-pCPT-cGMP since cGMP- and cAMP compounds show comparable lipophilicity. In general, it is recommended to start with a cyclic nucleotide concentration in the range of 1–10 μM and 10 min pre-incubation for most tissue and cell culture conditions. The immediate effects of an analog are most likely due to unspecific extracellular actions such as binding to surface receptors (Do et al. 2007).

In platelets, the intracellular PKG Iβ concentration is around 7 μM [respectively 14 μM taking into account the two binding sites (Eigenthaler et al. 1992)]. Therefore, activation of PKG in platelets will require extracellular cyclic nucleotide analog concentrations in the range of 100–200 μM.

For highly lipophilic compounds (relative lipophilicity >30) the use of serum-free medium during pre-incubation is advised. This will markedly reduce the complexation (and therewith immobilisation) of the cyclic nucleotides into lipid vesicles in the medium.

1.2 K-Serie Inhibitors

In the late 1980s a series of new protein kinase inhibitors based on the structure of staurosporine, an indole carbazole, were developed. Among these, KT5823 inhibited PKG with an apparent K_i of 0.234 μM whereas PKA is inhibited at concentrations >10 μM (Kase et al. 1987; see also Table 3). Caution is advised as these

Table 3 Apparent inhibition (K_i) and dissociation (K_d) constants. The cyclic nucleotide inhibition constants (K_i) for PKA I and II holoenzymes and PKG kinases Iα, Iβ and II were determined *in vitro* with increasing cGMP-analog concentrations competing for the natural activators cAMP, respectively cGMP. For determination of Epac dissociation constants (K_d) efficiency of cGMP-analogs to compete with [^{3}H]-labeled cAMP for binding to Epac-1 was measured

Inhibitors	PKG Iα K_i (μM)	PKG Iβ K_i (μM)	PKG II K_i(μM)	PKA I K_i(μM)	PKA II K_i(μM)	Epac K_d(μM)
Rp-cGMPS	20[a]	15[b]	0.5[b]		20[a]	
Rp-8-Br-cGMPS	3[b]	15[b]			20[b]	
Rp-8-pCPT-cGMPS	0.5[a]	0.6[a]	0.5[a]		8.3[a]	49[c]
Rp-8-Br-PET-cGMPS	0.035[d]	0.03[d]	0.9[c]			>100[c]
KT-5823	0.23[e]				>10[e]	
H89	0.5[e]				0.05[e]	
DT-2	0.012[f]				16.5[f]	
DT-3	0.025[f]				493[f]	

[a] Pöhler et al. 1995; [b] E. Butt unpublished results; [c] Poppe et al. 2008; [d] Butt et al. 1995, [e] Hidaka and Kobayashi 1992; [f] Dostmann et al. 2000

compounds compete with ATP and the K_i values quoted here were determined only under in vitro assay conditions. In intact cells, where the substrate (ATP) concentration is much higher, the compound may affect many other ATP- or even nucleotide-binding enzymes. For example, GSK3β and PRAK are inhibited by approximately 50% at 10μM KT5823 (Bain et al. 2003).

Several studies in which KT5823 was used in intact cells failed in observing a specific inhibition of protein kinase G (Wyatt et al. 1991; Burkhardt et al. 2000). As in most experiments the compound is used as a negative control to exclude the involvement of PKG in a specific signalling pathway, a second control experiment using a cGMP-analog, either as activator or inhibitor, is strongly recommended.

1.3 DT Inhibitors

Pseudosubstrate domains resemble protein kinase phosphorylation sites albeit the phosphate acceptor serine or threonine residue is replaced by alanine. For PKA a heat stable physiological protein kinase inhibitor (PKI) with a K_i of approximately 3 nM containing a pseudosubstrate region between residues 15 and 22 (RTGRRN**A**I) is known (Ashby and Walsh 1973). For PKG nothing comparable was discovered in the last 30 years. Therefore, synthetic oligopeptides were screened to identify selective substrate inhibitors that presumably do not interfere with ATP binding. The peptides are fused to a 13 amino acid long polypeptide of the *Drosophila* transcription factor *Antennapedia* (YGRKKRRQRRRPP- LRKKKKKH) to generate DT-3 or to the *HIV-1* Tat protein RQIKIWFQNRRMKWKK-LRKKKKKH to synthesize DT-2 and

to allow internalization in living cells (Dostmann et al. 2000). With K_i values of 0.025 and 0.012 μM, respectively, the data are in the range of the cyclic nucleotide inhibitor Rp-8-Br-PET-cGMPS (Table 3). DT-2 and DT-3 have been used in several studies with vascular smooth muscle cells (VSMC) (Taylor et al. 2004; Krieg et al. 2005; Zhou et al. 2007) and mesangial cells (Wang et al. 2007) to examine the effect of PKG.

Nevertheless, the peptides failed to inhibit PKG in intact platelets (E. Butt, unpublished results) – maybe due to fast degradation by trypsin, a protease that cleaves after arginine and lysine.

1.4 PKG siRNA

Another possibility to examine the role of PKG in cells, apart from pharmacological inhibitors, is the down-regulation of protein expression by small interfering RNA (siRNA) (Lenz 2005; Chang et al. 2006). With this method Christensen and Mendelsohn investigated the relative role of PKG Iα and Iβ in cGMP-mediated inhibition of $[Ca^{2+}]_i$ in VSMC (Christensen and Mendelsohn 2006) while Zhou and co-workers analysed the expression of SMC phenotype protein markers in dependency on PKG expression (Zhou et al. 2007). In both studies the PKG protein level was only suppressed by 75% or 85%, respectively and PKG over-expression was used in addition to reverse and verify the results. Whether siRNA oligonucleotides or even transgenic viral gene silencing can replace the knockout mice models in the future will depend on specificity and completeness of the PKG knock down.

Predesigned validated siRNA that has been functionally tested for PKG knockdown efficiency $\geqslant$70% is commercially available from several companies (e.g. Qiagen, Dharmacon).

1.5 PKG Control Substrates

Controlling the presence and activity of endogenous PKG in cells is an important aspect of attributing functions to PKG, particularly since these proteins are down-regulated in many primary cell lines upon passaging (Boerth et al. 1997) or are not expressed at all (Draijer et al. 1995). The presence of PKG can be determined by specific antibodies; however, the presence does not always correlate with function. Activation of PKG can be assessed by analyzing intracellular phosphorylation of the PKG specific substrate PDE5 (Rybalkin et al. 2002) or the PKG selective phosphorylation site in the protein VASP (Butt et al. 1994). VASP is phosphorylated by both PKA and PKG at Ser-157 and Ser-239, respectively, with a preference for Ser-239 phosphorylation by PKG, while Ser-157 is more rapidly phosphorylated by

PKA. The PKG selective phosphorylation can be detected by the 16C2 antibody (Smolenski et al. 1998).

In addition to VASP phosphorylation, PDE5 phosphorylation at Ser-92 is an effective monitor of intracellular PKG activation. Although PDE5 is phosphorylated by PKA and PKG in vitro, in intact cells the protein is tightly regulated by cGMP binding and PKG phosphorylation (Lin et al. 2006). PDE5 phosphorylation associated with PKG activation has been analyzed in several studies including smooth muscle, human platelets and mouse cerebellum (Mo et al. 2004; Rybalkin et al. 2002; Shimizu-Albergine et al. 2003). A more detailed analysis of tissue and cell type suitability of PKG, VASP and PDE5 is summarized by Lohmann and Walter (2005).

1.6 Conclusion

Despite 30 years of research, at present no perfect PKG activator or inhibitor is available. Even though the discussed compounds can be quite valuable tools in analyzing PKG signaling pathways, each individual cell type requires careful controls for possible "off target" effects such as inhibition of PDEs, stability of the compound or activation of other cyclic nucleotide binding proteins and enzymes to avoid misinterpretation.

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cGMP-Dependent Protein Kinase as a Modifier of Behaviour

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Abstract The importance of cGMP-dependent protein kinase (PKG) to the modulation of behavioural phenotypes has become increasingly clear in recent decades. The effects of PKG on behaviour have been studied in diverse taxa from perspectives as varied as ethology, evolution, genetics and neuropharmacology. The genetic variation of the *Drosophila melanogaster* gene, *foraging (for)*, has provided a fertile model for examining natural variation in a single major gene influencing behaviour. Concurrent studies in other invertebrates and mammals suggest that PKG is an important signalling molecule with varied influences on behaviour and a large degree of pleiotropy and plasticity. Comparing these cross-taxa effects suggests that there are several potentially overlapping behavioural modalities in which PKG signalling acts to influence behaviours which include feeding, learning, stress and biological rhythms. More in-depth comparative analyses across taxa of the similarities and differences of the influence of PKG on behaviour may provide powerful mechanistic explications of the evolution of behaviour.

Keywords: cGMP-dependent protein kinase · Behaviour · Natural genetic variation · Evolution · Pleiotropy · Plasticity · Foraging · Learning and memory · Stress · Rhythms

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1 A Natural History of PKG and Behaviour

The role of PKG in the modification of behavioural phenotypes arose initially out of ethological studies investigating natural populations of larval *Drosophila melanogaster*. Although *D. melanogaster* had been a model for the genetic dissection of phenotypic traits since the early 20th Century, it was not until the 1970s that investigations specifically addressed larval foraging behaviour (Sewell et al. 1975). By the late 1970s it became clear that larval behaviour had an important effect on resource utilization, competitive ability and fitness, prompting ethological and genetic descriptions of larval foraging strategies (Sokolowski 1980). Early studies showed that larvae differed in their foraging strategies displaying a phenotypic dimorphism (Sokolowski 1980; Bauer and Sokolowksi 1985). Individuals were dubbed either rovers, who traveled over a large area while foraging, or sitters, who covered a relatively smaller area. This distinction was quantitatively described by a bimodal distribution of the distance traveled while foraging (foraging trail-length). Later it was shown to arise from variation at a single locus named *foraging (for)* which encodes a cGMP-dependent protein kinase (PKG) (de Belle et al. 1989; Osborne et al. 1997). The polymorphism is not expressed when rovers and sitters are on a non-nutritive substrate (i.e. their foraging trail-lengths do not differ significantly on agar) suggesting a role for PKG signalling in the processing of a complex behaviour induced by a nutritive environmental stimulus (Douglas et al. 2005).

Several investigations indicated that the dimorphic behaviour exists in nature in the area of Toronto, Canada at about 70% rovers to 30% sitters (Sokolowski 1980, 1982; Sokolowski et al. 1997). Furthermore, when flies were collected from various microhabitats of a pear orchard, the rover and sitter variants were found foraging together within the fruit but preferred different pupation sites (Sokolowski 1986). While sitters pupated on the fruit, rovers were found to pupate off of the fruit.

The ecologically important process of density dependence was also found to be involved in changes of *for* allele frequencies (Sokolowski et al. 1997). In high density lab conditions rover larvae are preferentially selected whereas in low densities sitters predominate. More recent investigations suggest that the polymorphism may, in part, be maintained by balancing selection through a mechanism of negative frequency dependent selection under larval competition (Fitzpatrick et al. 2007).

Intriguingly, despite the strong association between the rover/sitter behaviour and *for*, the phenotypes are plastic when exposed to varying environmental parameters (Graf and Sokolowski 1989). For instance, expression of the behavioural polymorphism was found to be conditional on the distribution and abundance of food in the environment (Sokolowski et al. 1983; Kaun et al. 2007a). Specifically, food deprived rover larvae behave as sitters and exhibit sitter-like PKG activity (Kaun et al. 2007a). This is of particular interest since *D. melanogaster* larvae display habitat selection which has both heritable and plastic components (Rodriguez et al. 1992).

The first genetic analyses of larval foraging indicated that the behavioural polymorphism could be attributed to genetic properties on the second chromosome where the rover phenotype was found to be dominant to the sitter one (Sokolowski 1980). Subsequent analyses of isogenic rover and sitter strains indicated that the path

length phenotypes are autosomally inherited in a pattern indicative of a single gene influence (de Belle and Sokolowski 1987). Although a minor X-chromosome effect was observed in females, these analyses failed to detect other hereditary influences such as the Y-chromosome, transient maternal factors, permanent cytoplasmic factors or their interactions. Efforts to localize the genetic influence underlying the rover/sitter polymorphism were impeded by the modifying effects of phenotypic markers used in standard recombination mapping. To overcome this problem, de Belle and Sokolowski (1989) used compound reverse metacentric chromosomes which allowed for the localization of the allelic polymorphism to the left arm of the second chromosome of *D. melanogaster*. The lethal tagging technique was then used to genetically map the behaviour to chromosomal location 24A3-5 (de Belle et al. 1989, 1993). The technique involved mutagenizing rovers that were subsequently screened for the induction of both pupal lethality and sitter behaviour. The pupal lethality allowed for the subsequent mapping of the behavioural alteration. A number of mutations failed to complement for pupal lethality or sitter behaviour and they were readily mapped on the basis of their lethality to position 24A3-5 after which the behavioural alteration was also mapped to this position. The gene was named *foraging* (*for*) where rover alleles were designated as for^{R} and sitter alleles as for^{s}. Thus, the lethal tagging approach allowed for finer mapping of the behavioural polymorphism without the interference of marker genes and genetic backgrounds introduced in recombination mapping. The technique also provided mutant alleles for future studies.

The *for* gene was mapped within the region of a *Drosophila* cGMP-dependent protein kinase gene called *dg2*; cloning of *for* confirmed that it is synonymous with *dg2* (Osborne et al. 1997). Evidence for this includes the findings that wild-type for^{s} and sitter mutants, made on a rover background (for^{s2}), had lower PKG activity and *dg2* transcript levels in larval central nervous systems (CNS) and adult heads compared to wild-type for^{R} animals. Additionally, and perhaps most importantly, rover-type PKG activity and foraging behaviour resulted from increasing the level of *for/dg2* in transgenic sitter larvae (Osborne et al. 1997).

The natural allelic variation resulting in differing levels of PKG has allowed for the examination of variation in a single major gene affecting an ecologically relevant set of behavioural phenotypes from an evolutionary perspective (Douglas et al. 2005). Early studies were driven by unique lines of inquiry which have since had parallels in studies of other taxa such as nematodes, bees and ants (de Bono and Bargmann 1998; Ben-Shahar et al. 2002, 2003; Ingram et al. 2005). The implication of PKG in affecting diverse behavioural phenotypes in divergent species has brought increased attention to the role of PKG pathways in the evolution of behaviour (Fig. 1; Table 1).

The study of PKG in mammals, and in particular NO-cGMP-PKG signalling, preceded much of the work in invertebrates. However, many of the pharmacological and neuropharmacological studies only began to examine behavioural effects of PKG signalling more recently (Hofmann et al. 2006). The following sections will review these studies and consider the implications of their results for future research looking at the role of PKG signalling in the evolution and modulation of behaviour.

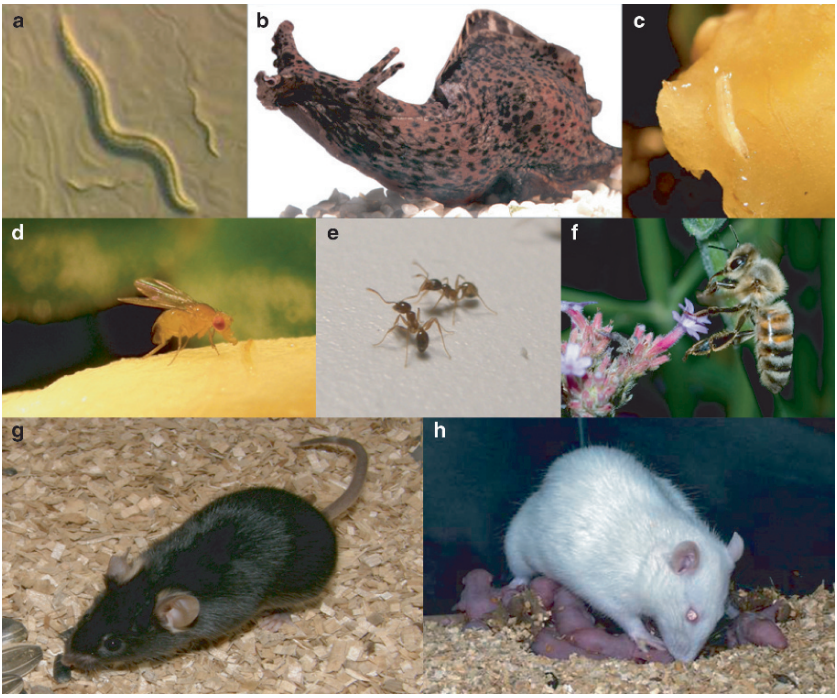

Fig. 1 cGMP-dependent protein kinase modulates behaviour in a variety of species. **a** *Caenorhabditis elegans* on a bacterial lawn **b** *Aplysia californica* **c** *Drosophila melanogaster* larva foraging on fruit **d** *Drosophila melanogaster* adult extending proboscis to feed **e** two worker ants of *Phidole pallidula* whose primary job is to forage for the colony **f** the honey bee, *Apis melifera* foraging for the hive **g** the lab mouse *Mus musculus* **h** the lab rat *Rattus norvegicus*. Photo credits: **a** Mario de Bono, **b** John H. Byrne, **c, d, e, g** Christopher J. Reaume, **f** Zachary Huang, Michigan State University, www.beetography.com, **h** Members of Alison Fleming's Lab

2 PKG and Food

The effects of *for* are not limited to larval foraging behaviour. Early descriptions of the rover/sitter polymorphism suggested that *for* is pleiotropic and that the allelic influences of PKG activity have resulted in various correlated traits including behavioural ones (Table 1). Initial investigations of these correlated effects demonstrated that rover females had no preference for oviposition site whereas sitters preferred to oviposite on sites that were inhabited by larvae (Sokolowski 1987). Descriptions of adult space-use and food-related behaviours showed that differences between for^R and for^s genotypes extend to life-history characteristics beyond the larval stage (Nagle and Bell 1987; Pereira and Sokolowski 1993). For instance, rovers were found to move farther away from a sucrose source following feeding.

More recent ecological and evolutionary studies have implicated the rover/sitter behaviours, and therefore PKG, in their investigations. For example, populations

Table 1 cGMP-dependent protein kinases (PKG) affect a variety of behaviours in diverse taxa

Species	Gene	Influence of PKG on Behaviour	References
C. elegans	*egl-4*	Food-related behaviours	Fujiwara et al. (2002); L'Etoile et al. (2002)
		Behavioural quiescence	Raizen et al. (2008)
Drosophila melanogaster	*for/dg2*	Larval forgaging path-length	Sokolowski (1980)
		Adult movement post-feeding	Nagle and Bell (1987); Pereira and Sokolowski (1993)
		Adult response to yeast	Shaver et al. (1988)
		Habituation	Engel et al. (2000)
		Sucrose response and habituation	Scheiner et al. (2004); Belay et al. (2007)
		Adult aversive olfactory associative learning and memory	Mery et al. (2007)
		Stress tolerance	Dawson-Scully et al. (2007)
		Larvae nutrient acquisition and absorption	Kaun et al. (2007a)
		Larval olfactory reward conditioning	Kaun et al. (2007b)
		Sleep	Raizen et al. 2008
		Visual pattern memory, operant learning	Wang et al. (2008)
Apis melifera	*Amfor*	Transition from nurse to forager	Ben-Shahar et al. (2002)
		Phototaxis	Ben-Shahar et al. (2003)
Pogonomyrmex barbatus	*Pbfor*	Worker and forager	Ingram et al. (2005)
Mus musculus	*cGKI*	nociception responses	Schmidt et al. (2002); Tegeder et al. (2004)
	cGKI	Cerebellar LTD	Feil et al. (2003)
	cGKI	Hippocampal L-LTP	Kleppisch et al. (2003)
	cGKI	Addiction	Jouvert et al. (2004)
	cGKII	Circadian rhythmicity	Oster et al. (2003)
	cGKII	Addiction and anxiety	Werner et al. (2004)
Rattus norvegicus	*cGKII*	Circadian rhythmicity	Tischkau et al. (2004)

selected for rapid development also expressed reduced foraging (Prasad et al. 2001). Investigations directed at examining adult space-use and habitat selection in which larval foraging distances were found to positively relate to lateral movements of adults also suggested that *for* may be involved in dispersal distances from food (Stamps et al. 2005). Given that an ever increasing number of pleiotropic effects of the *for* polymorphism were being discovered, the next obvious step was to try to understand what behavioural modalities and underlying circuitry PKG signalling was acting in to influence behavioural differences. Olfaction is an important and genetically well-studied modality involved in food-searching, foraging and feeding

behaviours. Shaver et al. (1998) asked whether rovers and sitters differed in their olfactory response (attraction). While larvae did not differ, sitter adults had a greater tendency to move towards a yeast source than did rover adults.

The *for* polymorphism affects larval food acquisition and, as with the foraging trail-length phenotype, its effects are sensitive to environmental variation (Kaun et al. 2007a). The behavioural effects of the natural variation in *for* may have an underlying metabolic basis since larvae with for^{R} and for^{s} alleles differ in their nutrient acquisition and absorption. For example, compared to for^{s}, for^{R} larvae have lower food intake, higher levels of glucose absorption and preferential allocation of glucose to lipids. Some of these differences can be modified by rearing larvae in conditions with lower food quality or availability. Such manipulations result in an overall rise in food intake in all strains but in these conditions rover intake no longer differs from that of sitters. Interestingly, for^{R} larvae maintain higher absorption efficiency but also have more rapid development and higher survivorship compared to for^{s} and for^{s2} when food is limited (Kaun et al. 2007a).

Recent studies have investigated members of the upstream signalling cascade thought to affect PKG-mediated behavioural phenotypes. For instance, Riedl et al. (2005) assayed the foraging behaviour of *dgcα1* mutants. These mutants are deficient in a soluble guanylyl cyclase gene and were therefore predicted to produce less cGMP, leading to lower PKG activation and thus more sitter-like foraging behaviour. Contrary to expectations, the mutants expressed both increased PKG activity and greater foraging trail-lengths compared to controls, in both rover (for^{R}) and sitter (for^{s}) genetic backgrounds. Perhaps these results are not really surprising given that cGMP signalling has a substantial amount of inbuilt degeneracy with upwards of 12 genes with known or predicted guanylyl cyclase activity in *Drosophila* (Riedl et al. 2005). DNA microarray analyses of transcriptional differences between *dgcα1* mutants and controls were performed and comparisons were made on both rover and sitter genetic backgrounds. Although many genes showed differential transcription, few differences were common to both backgrounds. Such results demonstrate the importance of genetic background when dissecting the signalling pathways involved in complex behavioural traits.

Complementary to reverse genetic approaches like that of Riedl et al. (2005), forward genetic approaches have been used to identify genetic modifiers of larval foraging behaviour (Pereira et al. 1995). *Chaser* (*Csr*), a dominant suppressor of for^{R}, was uncovered in a gamma mutagenesis screen of sitter flies. Although *Csr* has been mapped to regions 95F7-96A1 on the third chromosomes of *D. melanogaster*, the molecular basis of its modifying effects have not yet been determined. Shaver et al. (2000) used a mutagenesis screen to identify new genes that affect larval foraging behaviour. Once cloned, some of these genes may identify new members of the PKG signaling pathway important to larval foraging behaviour.

The influences of PKG on food-related behaviours are not restricted to *D. melanogaster* (Table 1). Investigations have been undertaken in several hymenopteran species which differ significantly in their life-history and social behaviour compared to *D. melanogaster* (Ben-Shahar et al. 2002; Ingram et al. 2005). In the honeybee, *Apis melifera*, PKG levels are associated with a temporal

polyethism which is important to the maintenance of the colony. Briefly, division of labour in *A. melifera* is described by the individual being a part of either a young cohort which act as nurses inside the hive or an older cohort (>3 weeks in age), which forage and defend outside the hive (Beshers et al. 2001). Ben-Shahar et al. (2002) found that transcript levels of the *for* orthologue, *Amfor*, as well as PKG activity were greater in foragers compared to the nurses. These effects were also causally demonstrated by pharmacological activation of cGMP (to increase PKG activity) which resulted in precocious foraging behaviour in young bees. Another study looking at red harvester ants (*Pogonomyrmex barbatus*) found similar, yet seemingly opposite, effects of the *for* orthologue of this species (*Pbfor*). In *P. barbatus*, mRNA levels of *Pbfor* were greater in young worker brains compared to those of foragers (Ingram et al. 2005).

Preliminary phylogenetic analyses suggest that the role of PKG in modulating food-related phenotypes may have a common evolutionary origin (Fitzpatrick and Sokolowski 2004). However, the specific circuitry and signalling underlying the effects of PKG expression and activity in diverse taxa are most likely dependent on aspects of the anatomy and life-history of the organism in question and so may provide interesting examples of evolutionary co-option from a behavioural perspective (Toth and Robinson 2007).

Another well-described organism that has provided insight into the role of PKG in the modification of food-related behaviour and physiology is the nematode *Caenorhabditis elegans* (Table 1). The *C. elegans* PKG mutant, *egl-4*, was first described phenotypically from an observed defect in egg-laying behaviour. Not unlike what has been found with *for* in *D. melanogaster, egl-4* has since been shown to affect diverse aspects of *C. elegans* biology including physiology, development, and behaviour.

Daniels et al. (2000) implicated PKG in the TGF signalling pathway which affects the dauer developmental stage of nematodes. Their results suggest that PKG signalling acts to modulate responses to sensory cues which are likely involved in both development and diverse behaviours including foraging, egg-laying, and stress responses. The effects of *egl-4* were directly implicated in the regulation of food-related behaviour by *in vivo* descriptions of neuronal signalling mechanisms (Fujiwara et al. 2002; L'Etoile et al. 2002).

C. elegans foraging behaviour is described by dichotomous behavioural states which are superficially similar to those found in *D. melanogaster* larvae. An individual will alter between two states called dwelling, in which speed is low and turning frequent, or roaming in which speed is high and turning infrequent. Ciliated sensory neurons involved in modulating these behavioural states, by inhibition of locomotion in the presence of food, were shown to have downstream effects partially mediated by PKG (L'Etoile et al. 2002). In contrast to expectations from observations in flies and bees, food-related locomotion and PKG are inversely related in *C. elegans*. Specifically, *egl-4*-PKG appeared to have a negative regulatory function in sensory neurons controlling this behaviour. The PKG signaling pathways involved in the behavioural effects of *egl-4* are not yet known. Nonetheless, a dominant mutation in *egl-4* causing increased gene activity has confirmed the role of

PKG in *C. elegans* locomotion and feeding and promises to be a valuable tool for elucidating the relevant signaling pathways (Raizen et al. 2006).

Overall, PKG affects feeding behaviours in a number of organisms but the exact details of these effects are likely to vary from species to species. However, a number of the developmental and behavioural effects of PKG in *C. elegans* appear to share commonalities with those of *D. melanogaster* (L'Etoile et al. 2002; Hirose et al. 2003; Raizen et al. 2006). Interestingly, as in *Drosophila*, behaviours affected by PKG display plasticity which may be modulated by environmental stimuli (Fujiwara et al. 2002).

3 PKG and Memory

Based on PKG's general neuromodulatory role, it is perhaps not surprising that it has been implicated in affecting phenotypes and mechanisms underlying learning and memory. There are many examples across divergent species of the interaction between learning, memory and food-related behaviours. For example, taxa as diverse as insects, birds, and mammals can anticipate the spatial and temporal availability of food in order to alter their foraging strategies (Shettleworth 2001; Boisvert and Shery 2006). In this section, we review studies in *D. melanogaster* as well as in mammalian models that suggest an important role for PKG signalling in the mechanisms of behaviours associated with learning and memory.

In *D. melanogaster, for*-PKG has been shown to affect habituation-related behaviours (Engel et al. 2000; Scheiner et al. 2004). Habituation is a well-studied form of non-associative learning in which a behavioural response is reduced or extinguished following repeated stimulation (Thompson and Spencer 1966; Castellucci and Kandel 1974). To assess the role of PKG in habituation and dishabituation, Engel et al. (2000) examined the stimulus-dependent response decrement of the long-latency giant fiber jump-and-flight escape response of intact tethered *for* genetic variants. While this escape response can be induced by a visual startle reflex, in this study the response pathway was electrically stimulated using electrodes implanted through the animal's eyes (Engel and Wu 1996). Levels of PKG were found to affect several parameters of the response. Specifically, rovers had a slower response decrement, a weaker reversal of the response decrement post-stimulus and stronger reversal of the response decrement evoked by a novel stimulus. Thus, natural genetic variation influencing PKG activity was shown to affect the neuronal plasticity of the giant-fiber circuit which underlies a non-associative sensorimotor behaviour (Engel et al. 2000). The precise mechanism or signalling pathways underlying these effects were not examined.

The role of *for*-PKG in habituation extends to a well-known phenotype in the invertebrate learning and memory literature: the proboscis extension response (PER). PER has long been used to assess sucrose responsiveness (SR) which itself correlates to other phenotypes such as odour preferences and phototaxis and is thought to be indicative of responsiveness in multiple behavioural modalities (Scheiner et al. 2004). Interestingly, SR correlates with the division of labour amongst foraging bees

(Scheiner et al. 2003). In these experiments, a drop of solution containing some stimulant, such as sugar, is applied to taste sensilla on the leg and the resultant proboscis extension response (PER) events are noted.

The for^{R}, for^{s} and for^{s2} strains, with their differing PKG activities, showed differences in sucrose responsiveness and habituation of the PER to repeated sucrose stimulations. The for^{R} line, with higher PKG activity, had higher levels of responsiveness to sucrose than did the natural sitter and the sitter mutant strains. Additionally, when flies with similar sucrose responsiveness were compared, rovers habituated more slowly and had less generalization of habituation than did sitter flies. The relationship of *for*-PKG to sucrose responsiveness has recently been supported using transgenic flies (Belay et al. 2007). A pan-neuronal driver (*elav-gal4*) was used to express *for* transcripts in neurons of the adult fly brain and this resulted in an increase of sucrose responsiveness in sitters.

Recently, rovers and sitters have been shown to differ in their short and long-term memory abilities in an associative olfactory learning paradigm (Mery et al. 2007). Specifically, Mery et al. (2007) found that sitters have poorer short-term memory but better long-term memory than rovers. Further, GAL4 drivers used to direct *for* expression to the mushroom bodies (MB) of for^{s} flies induced rover-like learning performance. The MB are central to olfactory learning processes in *D. melanogaster* (Heisenberg 2003) and the *for*-PKG protein has been localized in the MB (Belay et al. 2007). An earlier study of brain structure-function relationships of *for* expression found, however, that MB ablation in larvae did not affect the larval rover/sitter path-length phenotype (Osborne et al. 2001).

In larvae, Kaun et al. (2007b) showed that olfactory conditioning was also significantly influenced by *for* expression in the MB. Using an olfactory reward-conditioning paradigm, rover and sitter larvae were found to differ phenotypically. The for^{R} larvae, which exhibit greater PKG activity, showed faster memory acquisition and longer retention. Once again, the influence of PKG on the phenotype was sensitive to an environmental stimulus where in this case, an increase in the number of conditioning trials removed the phenotypic differences between the *for* genotypes (Kaun et al. 2007b).

Findings on *for*-related learning suggests that rovers and sitters may use different foraging tactics in nature and that *for* may be involved in an evolutionary trade-off between short-term learning performance and long-term memory (Mery et al. 2007). Because rovers and sitters are known to exploit different microgeographic habitat types in nature (Sokolowski 1986; Sokolowski and Carton 1989), it may be that *for*-PKG expression in the MB plays an important role in modulating decision-like processes that involve learning, memory and habitat selection. Indeed, it has been shown that flies without the MB are unable to suppress their memory from previous experience (Liu et al. 1999). Thus, the MB may be involved in the balance between maintaining an existing behaviour and switching to a new one. Taken together, these results and observations suggest that PKG signalling affecting several learning- and feeding-related behavioural phenotypes occur in separate and distinct neuronal circuits that underlie multiple modalities which can be modified by environmental stimuli.

Long-term potentiation (LTP) and long-term depression (LTD) are activity-dependent changes in synaptic transmission that are thought to play an important role in diverse forms of learning and memory. Briefly, these processes of synaptic plasticity can be described as the long-term increase (LTP) or decrease (LTD) of synaptic strength after activity-dependent changes in the strength and frequency of stimulation (Chen and Tonegawa 1997). Hippocampal LTP has been implicated in spatial and contextual learning, whereas cerebellar LTD is known to contribute to discrete motor learning. Research has suggested that hippocampal LTP and cerebellar LTD are both modulated through NO-cGMP-dependent signalling mechanisms (Chen and Tonegawa 1997; Boyden et al. 2004). As in *Drosophila*, vertebrate genomes contain two known PKG genes: cGKI (homologous to *Drosophila for/dg2*) and cGKII (homologous to *Drosophila dg1*). The mammalian PKG isoform, cGKIα, while having a variety of physiological (Hofmann et al. 2006) and neuronal (Feil et al. 2005) effects, exists in high concentrations in cerebellar Purkinje cells (PCs), which are involved in the modulation of various types of motor learning. PCs relay inhibitory signals from the cerebellum and receive a number of excitatory neuronal inputs. Both the induction and inhibition of LTD has been shown to be affected by upstream signalling components of PKG; however, it is thought that PKG serves a key function in cerebellar LTD. Additionally, pharmacological approaches using enzyme inhibitors with varying specificities suggested that PKG is involved in cerebellar LTD (reviewed in Feil et al. 2005).

A conditional knockout of cGKI in the PCs of mice showed that PKG is involved in cerebellar LTD (Feil et al. 2003). These experiments demonstrated that a lack of cGKI in the PCs led to a nearly complete loss of LTD at the synapses of PCs and granular layer parallel fibers (PF-PC synapses). Because the PCs are uniquely at the confluence of retinal and vestibular inputs, cerebellar LTD is thought to be involved in vestibular-ocular reflex adaptation (VOR). The exact mechanisms underlying cGKI's effect on cerebellar LTD has yet to be precisely elucidated (Feil and Kleppisch 2008).

The hippocampus is important for what might be a uniquely mammalian form of memory (Hawkins et al. 2006) known as explicit or declarative memory. Implicit memory is thought to underlie memory for motor skills and task-specific functions that do not invoke the need for conscious recall of a past experience, whereas explicit memory is thought to involve a more epistemic form of recall of, for example, specific places, objects, or past experiences.

Several upstream components of the NO-cGMP-PKG signalling pathway have been implicated in hippocampal LTP and, indeed, hippocampal neurons are known to predominantly express cGKIβ (Feil et al. 2005). Thus, a number of studies have been devoted to elucidating the role of NO-cGMP-PKG signalling in hippocampal LTP in hopes of gaining a better understanding of the mechanisms underlying explicit memory (reviewed in Feil et al. 2005; Hofmann et al. 2006) The role of PKG in hippocampal LTP was first implied by the observation that, following weak tetanic stimulation, hippocampal LTP was enhanced or suppressed in the presence of PKG activators and inhibitors, respectively (Zhuo et al. 1994). While mounting evidence suggests that retrograde signalling of NO via Ca^{2+}/calmodulin is important

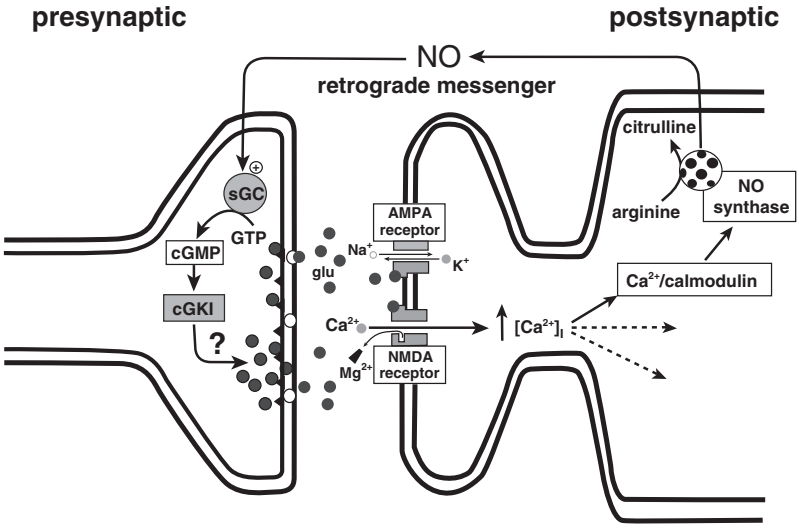

Fig. 2 A model of NO-cGMP-PKG signalling underlying LTP in Shaffer collateral/CA1 synapses of the hippocampus. Ca^{2+} influx into the postsynaptic neuron is mediated by NMDA receptors. An increase in Ca2+ concentrations in the postsynaptic neuron activates Ca^{2+}-calmodulin-dependent NOS. The retrograde action of NO then leads to increased neurotransmitter release in the presynaptic neuron via a NO-cGMP-PKG cascade (used with permission from Feil et al. 2005)

for the induction of LTP in Schaffer collateral/CA1 synapses of the hippocampus (Bolshakov and Siegelbaum 1995; Arancio et al. 2001), the downstream relationship to PKG has remained more elusive (Fig. 2). For instance, hippocampal LTP was found to be normal in mice lacking either one or both genes for cGKI and cGKII (Kleppisch et al. 1999).

More recent evidence suggests that PKG is an important target for NO/cGMP in this context given its effects on a protein synthesis-dependent late phase (L-LTP) form of LTP (Kleppisch et al. 2003). Using a hippocampus-specific cGKI knockout mouse line it was demonstrated that, while early-phase LTP (E-LTP) remained normal, L-LTP in the hippocampus was impaired following multiple episodes of strong theta burst stimulation. However, this synaptic effect does not appear to modulate the spatial learning and memory of the animal and L-LTP effects are only present in adult mice (Kleppisch et al. 2003). It remains to be seen, however, if this lack of correlation is due to the specific behavioural paradigms used in this study. That is to say, the effects of PKG on age-related L-LTP may extend to behaviours which have not yet been examined (Feil et al. 2005).

4 PKG and Stress

It is becoming increasingly clear that PKG signalling is important for modulating environmental stress. Although investigations of this type are emerging, PKG's roles in stress span developmental, physiological and behavioural phenotypes; however,

many of these biological influences may not be separable into discrete phenotypic outcomes. For instance, cGKIα expression patterns in the sensory cells of the dorsal root ganglion suggest that this isoform is involved in the development of sensory axons (Schmidt et al. 2002). Using cGKI-deficient mouse embryos, Schmidt et al. (2002) showed that PKG influences axonal branching of sensory neurons while also impairing nociceptive flexion reflexes. The influence of PKG signalling on nociceptive behaviour has focused primarily on NO and cGMP and the results have been mixed. Both positive and negative nociceptive responses have been attributed to NO-cGMP signalling (Tegeder et al. 2002; Vivancos et al. 2003). PKG has been more directly implicated in nociceptive behavioural responses in mammals using the response evoked by hind paw formalin injection. Conventional cGKI knockout mice were found to have lowered formalin-invoked licking behaviour than their wild-type counterparts (Tegeder et al. 2004). The elucidation of the precise role of NO-cGMP-PKG signalling in the nociceptive response is still in its infancy but the underlying mechanisms may be illuminated by existing research of *Aplysia* nociception (see Hofmann et al. 2006 for discussion).

Several lines of evidence have also implicated PKG in the behavioural effects of addictive drugs in mammals. Pharmacological manipulations which either stimulated cGMP production or activated PKG, as well as tissue specific overexpression of cGKIα by plasmid injection, were found to reduce cocaine-induced *egr-1* expression and also affect locomotor behaviour (Jouvert et al. 2004). These effects were reciprocally reversed by subsequent silencing or activation of PKG signalling following the initial treatment. Thus, NO-cGMP-PKG signalling is important for regulating cocaine-related effects on behaviour.

Similarly, NO-cGMP-PKG signalling has been implicated in anxiety-like behaviour as well as behavioural response to alcohol (Werner et al. 2004). When compared to wild-type mice, cGKII-deficient animals appeared to display augmented anxiety-like behaviour. Using a measure of the persistence of the hypnotic effect of ethanol, known as the loss of righting reflex, cGKII knockout mice were more resistant to the effects of ethanol. Furthermore, the cGKII mutants showed a greater preference for ethanol compared to wild-type mice (Werner et al. 2004).

Consistent with mammalian studies which suggest a role for PKG in stress resistance, recent studies of the *for* genetic variants in *D. melanogaster* suggest that a PKG pathway underlies synaptic response to thermal stress (Dawson-Scully et al. 2007). Cultured giant neurons and the larval neuromuscular junction (NMJ) of rovers and sitters were found to have dramatic physiologic polymorphisms (Renger et al. 1999). Particularly, cultured sitter neurons were found to have lower, more transient K^+ current conductance compared with the rover neurons. Pharmacological PKG inhibition of the rover neurons reduced K^+ conductance. Studies of heat shock-mediated protection of neural function in *Locusta migratoria* reported similar reductions in K^+ currents inducing thermotolerance of synaptic transmission (Ramirez et al. 1999) as well as central pattern generation (Newman et al. 2003).

Dawson-Scully et al. (2007) hypothesized that PKG acts as a regulator of neuronal thermotolerance and therefore mediates protection against heat-induced neuronal trauma. Both genetic and pharmacological manipulations indicate that

reductions in PKG or PP2A (protein phosphotase 2A) activity result in increased thermotolerance of synaptic transmission of the larval NMJ (Dawson-Scully et al. 2007). To assess the behavioural effects, *Drosophila* larvae were heated, along with their nutritive substrate, until cessation of larval moth hook movements was observed. Strains with lower PKG activity reached significantly higher temperatures before behavioural failure. Intriguingly, PKG or PP2A inhibition also provided strong thermotolerance of synaptic transmission in *D. melanogaster* and of a central circuit in *L. migratoria*, suggesting a conserved neuroprotective function of this pathway (Dawson-Scully et al. 2007) The role of PKG-PP2A signalling also appears to extend to other stress related pathways including hypoxia where low levels of PKG activity are, again, associated with increased tolerance to stress, in this case to anoxic conditions (Dawson-Scully pers. comm.).

5 PKG and Clocks

Circadian rhythmicity and molecular clock functions are highly conserved taxonomically and are important biological processes which interact with, or underlie, many physiological and behavioural processes (Dunlap et al. 2004). A number of studies have implicated kinases, including PKG, in the regulation of the circadian clock system (e.g. Comolli and Hastings 1999; Mathur et al. 1996; Tischkau et al. 2003; Agostino et al. 2007). In mammals, the suprachiasmatic nucleus (SCN), which is seated above the optic chiasm, receives retinal input from the optic nerve by means of the retinohypothalamic tract. The well-known feedback mechanism of the molecular clock is active in cells of the SCN where photosensory input entrains the circadian transcription/translation system (e.g. *Per, Tim, Clock, Bmal1*) to the organism's light/dark cycle.

NO-cGMP-PKG signalling has been implicated in circadian function by both pharmacological and genetic manipulations (reviewed in Golombek et al. 2004 and see also Hofmann et al. 2006). Indeed, significant phase delays of circadian rhythmicity can be caused by inhibition of clock-controlled increases in PKG activity (Tischkau et al. 2003). In mice, PKG appears to play a role in the modulation of the phase shift in which time-dependent exposure to light results in changes to the biological clock and of concomitant alterations in *mPer1* and *mPer2* expression (Oster et al. 2003). When compared to wild-type mice, differences in the onset of wheel running after a light pulse in cGKII-deficient mice suggested an aberration in circadian clock synchronization. While cGKII deficient mice were found to have 50% reductions in early night (CT14) phase delay compared to wild type mice, no difference in the late night (CT22) phase delay was found (Oster et al. 2003). Furthermore, Oster et al. (2003) found that the loss of cGKII resulted in a reduction of early night light-induced expression of *mPer2* while *mPer1* induction was elevated. cGKII was also implicated in phase advances of the diurnal domain of molecular clock function due to its observed effects on the positive feedback loop of the circadian transcription/translation system (i.e. *Bmal1* and *Clock*; Tischkau et al. 2004). SCN slice cultures were exposed to short-term cGKII inhibition using antisense

oligodeoxynucleotides which delayed rhythms of electrical activity as well as *Bmal1* mRNA production. Long-term inhibition of cGKII increased *Bmal1* mRNA and disrupted electrical activity rhythms. Pharmacological inhibition of cGKII resulted in the repetition of the last 3.5 hours of the cycle. Thus, PKG appears to be important for regulation and progression of the circadian cycle by influencing both the positive and negative arms of the clock feedback loop.

From a behavioural perspective, clock cellular mechanisms are an important part of the expression of various behaviours such as sleeping and feeding. Peripheral clocks, which reside outside of the SCN, can become asynchronous with the SCN due to environmental alterations. For example, when nocturnal animals are restricted to day-time feeding only, some peripheral clock organs become unsynchronized with the SCN and behaviour is altered to anticipate diurnal feeding times (Damiola et al. 2000; Stokkan et al. 2001; Horikawa et al. 2005). Interestingly, PKG has been implicated in the clock feedback mechanism of mammals, and in food intake in flies suggesting that food intake and circadian rhythms are intimately linked (Challet et al. 2003; Sarov-Blat et al. 2000; Lee et al. 2006; Mendoza et al. 2005; Challet 2007). Thus, PKG may play an important role in modulating complex composite behaviours through various signalling pathways of interacting circuits.

PKG has recently been implicated in clock-related behaviours in both *C. elegans* and *D. melanogaster* which have recently garnered a great deal of interest (Raizen et al. 2008). In mammals and flies it is called sleep; in worms it is called lethargus. The *C. elegans* homologue of *per* (LIN-42) is known to be expressed temporally with periods of lethargus. Raizen et al. (2008) demonstrated that lethargus has the crucial features which distinguish sleep, including reversibility, reduced-responsiveness, and homeostasis. Furthermore, they showed that PKG (*egl-4* and *for*) is a regulator of sleep-like behaviours in flies and worms. By comparing gain- and loss-of-function *egl-4* mutants, the authors demonstrated that PKG is associated with the extent of behavioural quiescence as well as its time-dependence. Raizen et al. (2008) then used the for^{R} and for^{s2} fly lines to ask whether the behavioural effect of PKG on sleep is evolutionarily conserved. And indeed, they found that the rover strain (for^{R}), with higher PKG activity, slept more than the sitter mutant on the rover genetic background (for^{s2}). Thus, in both species, PKG activity is positively associated with the amount of sleep that an animal displays.

6 Conclusion

While past studies of PKG biology were not as extensive as in other well-known kinases (Wang and Robinson 1997), recent research has shed light on its diverse roles in development, physiology and behaviour. Research into PKG and behaviour in invertebrate and mammalian models has progressed from rather divergent perspectives. Generally speaking, mammalian research has focused on elucidating neuronal PKG-related signalling pathways underlying behaviours. Concurrent invertebrate research has focused more on clarifying how natural genetic variation affecting PKG activity modifies behavioural phenotypes.

Commonalities emerging from many of these studies suggest that PKG is highly pleiotropic, acts through multiple modalities, and that its effects may be evolutionarily conserved. For instance, in flies, worms, and hymenopterans PKG affects food-related behaviours yet the phenotypic effects of PKG activity differs. This variation suggests that PKG function may have been utilized early on in metazoan evolution to affect food-related behavioural phenotypes while standing and/or new genetic variation has allowed for the specificities its effects to be modified in different taxa. Conversely, the effects of PKG on food-related behaviours may have evolved independently in these highly differentiated taxa. These hypotheses are becoming more testable with the increasing availability of genome sequences and bioinformatic tools.

The effects of PKG also appear to be modulated significantly by environmental context or stimuli, suggesting that this molecule is important for behavioural plasticity. This observation is supported by studies of the influence of PKG in the plasticity of caste-specific behaviours in hymenopterans (Ben-Shahar et al. 2002, 2003). Many interesting hypotheses emerge from these observations which are of interest to the evolution of behaviour and behavioural plasticity. For instance, one might ask whether biochemical properties of PKG distinguish it from other kinases allowing it to be more easily co-opted by selection for use in behavioural plasticity. One might also ask if the PKG-signalling that influences food-related behavioural plasticity functions along the same signalling pathways and homologous neural circuits in invertebrates.

Food-related behaviours present a good model for testing such hypotheses in invertebrates (Douglas et al. 2005). Nevertheless, comparisons across wider taxonomic gaps might also be approached by looking at PKG effects on learning and memory. PKG affects habituation, learning, and memory in flies, while also being involved in synaptic plasticity in mammals – i.e. processes that are thought to underlie learning and memory.

A model describing the role of PKG in modulating LTP in mammalian Schaffer collateral/CA1 synapses of the hippocampus suggests a critical role for NMDA receptors (NMDARs) (Fig. 2; Feil et al. 2005). Recently, NMDAR activation has been more directly implicated in mammalian learning through its effects on LTP and synaptic strengthening of the barrel cortex in the single-whisker experience protocol (Clem et al. 2007). Interestingly, NMDAR disruption in the ellipsoid body of *D. melanogaster*, a brain structure in which *for*-PKG is highly expressed, was shown to disrupt long-term memory consolidation of a Pavlovian olfactory learning paradigm (Wu et al. 2007). *for*'s presence in the ellipsoid body of *D. melanogaster*, a structure thought to be a control centre for a variety of behavioural outputs (Strauss and Heisenberg 1993), suggests that PKG may play an important role in the processing of information (Varnam et al. 1996; Belay et al. 2007). A recent paper examining operant learning indicated that PKG expression in the central complex is important for visual learning (Wang et al. 2008). Also, Mery et al. (2007) showed that PKG expression in the mushroom bodies is important for olfactory based associative learning. The tools used in these studies will allow investigators to determine whether PKG's influence on the cellular, synaptic and evolutionary bases of learning share common themes.

PKG affects development and physiology in both invertebrates and mammals but the implications of these effects for behaviour are still in early stages of understanding. Of primary importance to understanding the links between PKG and behaviour are descriptions of its interactive roles in development and of its distribution in tissues outside of the nervous system as well as in the peripheral and central nervous systems. Additionally, PKG may play important developmental roles in flies due to the lethality of several *for* alleles. Tools are now available to asses the relative influence of developmental and/or real-time gene expression of PKG on behaviour.

The use of increasingly well-known model organisms, advancements in sequencing and expression analyses, quantitative genetic approaches, neurophysiology, pharmacology and neuronal imaging are constantly advancing our understanding of behavioural effects of genetic architecture (Mackay 2004; Wagner et al. 2007), while great strides are being made in the understanding of the evolutionary processes which act on genetic variation for phenotypic traits (Mitchell-Olds et al. 2007). Exciting avenues for the future study of PKG and behaviour also include the functional analysis of enzymatic activity from an evolutionary perspective (Dean and Thornton 2007). Studies examining PKG signalling and behaviour in diverse taxa hold a great deal of potential in this regard.

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Part III
Clinical Applications

cGMP in the Vasculature

Barbara Kemp-Harper and Harald H.H.W. Schmidt

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Abstract Cyclic guanosine $3',5'$-monophosphate (cGMP) plays an integral role in the control of vascular function. Generated from guanylate cyclases in response to the endogenous ligands, nitric oxide (NO) and natriuretic peptides (NPs), cGMP influences a number of vascular cell types and regulates vasomotor tone, endothelial permeability, cell growth and differentiation, as well as platelet and blood cell interactions. Reciprocal regulation of the NO-cGMP and NP-cGMP pathways is evident in the vasculature such that one cGMP generating system may compensate for the dysfunction of the other. Indeed, aberrant cGMP production and/or signalling accompanies many vascular disorders such as hypertension, atherosclerosis, coronary artery disease and diabetic complications. This chapter highlights the main vascular functions of cGMP, its role in disease and the resulting current and potential

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therapeutic applications. With respect to pulmonary hypertension, heart failure and erectile dysfunction, as well as cGMP signal transduction, the reader is specifically referred to other dedicated chapters.

1 Vascular Physiology of cGMP

1.1 cGMP Signalling and Compartmentalisation

cGMP is generated in the vasculature via two main guanylate cyclases, the cytosolic soluble guanylate cyclase (sGC) and the membrane-bound particulate guanylate cyclase (pGC) (Münzel et al. 2003). sGC serves as a receptor for the biologically active gas nitric oxide (NO) (Friebe and Koesling 2003; Moncada and Higgs 2006). NO is produced endogenously in the vascular system via a family of NO synthases, namely the constitutive forms of endothelial NOS (eNOS) and neuronal NOS (nNOS) and the inducible form, iNOS (Moncada and Higgs 2006). Once generated, NO binds to the prosthetic heme group of sGC to induce a conformational change, breakage of the histidine-to-iron bond and activation of the enzyme (Friebe and Koesling 2003). In addition to NO, carbon monoxide (CO), generated via heme oxygenase (HO)-mediated degradation of cellular heme, also stimulates sGC, albeit to a lesser extent than NO (Brune and Ullrich 1987; Friebe et al. 1996; Sharma and Magde 1999) and modulates vascular function (Wang 1998; Kaczorowski and Zuckerbraun 2007; Li and Moore 2007). Moreover, sGC may also be activated by the reactive oxygen species, hydrogen peroxide (H_2O_2), which is generated predominantly via the dismutation of superoxide (O_2^-) (Ardanaz and Pagano 2006). Thus H_2O_2 has been reported to stimulate sGC and increase cGMP in the vasculature (Burke-Wolin et al. 1991; Fujimoto et al. 2001; Sato et al. 2003); other studies have refuted this concept (Thengchaisri and Kuo 2003; Gao et al. 2003; Iida and Katusic 2000).

pGC is the target of the natriuretic peptides (NPs), atrial (ANP), B-type (BNP) and C-type (CNP) natriuretic peptide and urodilatin (Ahluwalia et al. 2004b). ANP and BNP, released from the heart in response to hypervolaemia, circulate in the blood to regulate vascular function via the activation of the NP receptor termed GC-A (Ahluwalia et al. 2004b). Conversely, CNP is generated in the endothelium (Stingo et al. 1992) and acts in a local, paracrine fashion, targeting GC-B (Scotland et al. 2005). Similarly, urodilatin, which is secreted by the kidney, acts in the renal vasculature via activation of GC-A (Forssmann et al. 2001). The effects of NPs are limited by neutral endopeptidases (Soleilhac et al. 1992) which are found in high concentrations on the luminal surface of endothelial cells and via binding to the clearance receptor, GC-C, resulting in their subsequent internalisation.

Following production from either sGC or pGC, the effects of cGMP in vascular tissues are mediated via a number of effectors including cGMP-dependent protein kinases (cGKs, Hofmann et al. 2006), cGMP regulated phosphodiesterases (PDEs,

Conti and Beavo 2007) and cGMP-modulated cation channels (CNGs; Biel and Michalakis 2007, see chapter on CNGs). cGKs exist in three forms, cGKIα, cGKIβ and cGKII and are present in vascular cells, including vascular smooth muscle (cGKIα and cGKIβ, Hofmann et al. 2006), some types of endothelial cells (cGKI, Draijer et al. 1995) and platelets (cGKIβ, Hofmann et al. 2006). Indeed cGKI is a major mediator of vascular smooth muscle (VSM) relaxation with mice deficient in cGKI displaying impaired NO-cGMP dependent vasorelaxation (Pfeifer et al. 1998). cGK phosphorylates a number of target proteins, lowering intracellular calcium and mediating vasorelaxation (Hofmann et al. 2006). Thus evidence exists that vasodilation occurs through cGKIα-mediated activation of myosin light chain phosphatase (MLCP) and dephosphorylation of the myosin light chain (Surks et al. 1999; Wooldridge et al. 2004). cGKI can also activate MLCP indirectly via phosphorylation of Rho A and subsequent inhibition of Rho kinase (Ellerbroek et al. 2003). Alternatively, it has been suggested that cGKIβ interacts with IRAG (IP_3 receptor-associated cGKIβ substrate) resulting in inhibition of IP_3-induced intracellular Ca^{2+} release (Schlossmann et al. 2000; Geiselhöringer et al. 2004). Interestingly, the recent finding that restoration of either the Iα or Iβ isozyme of cGK in the smooth muscle of $cGKI^{-/-}$ mice, was able to lower intracellular Ca^{2+} and relax smooth muscle (Weber et al. 2007) suggests that neither the cGKIα-myosin phosphatase pathway nor the cGKIβ–IRAG pathway is absolutely essential for the regulation of vascular tone. Indeed additional cGK targets may include (a) the vascular smooth muscle large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}), phosphorylation of which leads to K^+ efflux from the cell, hyperpolarization and relaxation (Alioua et al. 1998; Fukao et al. 1999), (b) the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) regulator phospholamban, phosphorylation of which increases SERCA activity and promotes Ca^{2+} reuptake into the sarcoplasmic/endoplasmic reticulum (Raeymaekers et al. 1988; Koller et al. 2003) and (c) G protein signalling (RGS) proteins, phosphorylation of which leads to their activation and termination of signalling by Gq-coupled receptors for contractile agonists (Pedram et al. 2000; Tang et al. 2003). Thus multiple mechanisms underlie cGKI-mediated vasorelaxation, the nature of which may differ according to vessel origin, function and contractile status (Hofmann et al. 2006). Importantly an ubiquitous biochemical read-out for cGMP signalling in the vasculature is the cGKI-mediated phosphorylation of vasodilator-stimulated phospho protein (VASP; Ibarra-Alvarado et al. 2002; Melichar et al. 2004), although phosphorylation by cAK and PKC also occurs.

In addition to its vascular smooth muscle effects, cGK may also influence endothelial function particularly at the level of eNOS. Thus cGKI has been shown to phosphorylate and activate eNOS (Butt et al. 2000) and both cGKI and cGKII activate 6-pyruvoyltetrahydropterin synthase to produce the NOS cofactor, tetrahydrobiopterin (BH_4) (Scherer-Oppliger et al. 1999).

Interestingly, a recent study has identified cGKIα as a redox sensor, such that oxidation of its cysteine residues leads to its activation (Burgoyne et al. 2007). Specifically, oxidants such as H_2O_2 can directly activate cGKIα, independent of NO and cGMP, to elicit vasorelaxation. Such findings may account for the ability of H_2O_2 to

serve as an endothelium-derived hyperpolarizing factor in some vascular beds and provides an alternate, cGMP-independent mechanism of cGKI modulation in the vasculature.

PDEs, of which there are 11 families, degrade cAMP and/or cGMP, thereby regulating the intracellular concentration of these cyclic nucleotides and play an important role in compartmentalization of signalling (Conti and Beavo 2007). Eight of these PDEs hydrolyse cGMP and are present in arterial VSM cells (PDE 1–5, Wallis et al. 1999), venous VSM cells (PDE 1,4 and 5, Wallis et al. 1999) and platelets (PDE 2,3 and 5, Schwarz et al. 2001). PDEs can also be allosterically regulated by cGMP such that their activity is stimulated (PDE 2) or inhibited (PDE 3). In addition, cGKI phosphorylates and activates PDE5 to terminate the action of cGMP (Rybalkin et al. 2002; Mullershausen et al. 2003).

Interestingly, PDEs play an integral role in the compartmentalization of cGMP signalling. Indeed in cardiomyocytes pGC-derived cGMP is located close to the membrane and controlled by PDE2, whereas sGC-derived cGMP is distant from the membrane and controlled by PDE5 (Castro et al. 2006). Similarly in the systemic vasculature, PDE5 regulates sGC-derived but not pGC-derived cGMP as ANP-mediated vasodilation either in vitro or in vivo is not enhanced by the PDE5 inhibitor, sildenafil (Hobbs 2007; Kemp-Harper and Feil 2008). Moreover while PDE5 inhibitors will augment NO-cGMP signalling leading to severe hypotension, Ishikura et al. (2007) showed that sildenafil enhances vasodilatation by ANP in normal dogs without causing hypotension. Thus PDEs may be vital in forming intracellular signalling microdomains and conferring specificity to the effects of NPs, NO and PDE inhibitors in the vasculature. Compartmentalization of cGMP in the vasculature may also occur independent of PDEs. Using activation of CNG channels as a readout of cGMP activity in VSM, ANP induces activation of CNG channels more readily than NO (donated from SNAP), despite NO triggering higher levels of total cellular cGMP accumulation (Piggott et al. 2006). Similarly utilising non-FRET based cGMP biosensors named FlincGs in VSM, ANP has been shown to elicit submembrane elevations in cGMP whereas NO (donated from DEA/NO) caused a global increase in cGMP (Nausch et al. 2008). In this instance, however, the PDE5 inhibitor, sildenafil converted the ANP-mediated increase in cGMP to a global signal. Taken together these findings provide further evidence that cGMP signals are spatially segregated within cells.

In addition to PDEs, cGMP action may also be terminated by being transported out of the cell via a multidrug-resistant protein (Jedlitschky et al. 2000). However, this mechanism appears to operate only for cGMP generated by NP-pGC signalling, suggesting again relevant compartmentalisation of different cGMP generating and signalling systems and cGMP extrusion as a potential biomarker for NP signalling. As mentioned previously, cGMP may also transduce its effects via non-cGK/PDE mechanisms such as CNGs (Biel and Michalakis 2007, see chapter on CNGs). Endothelial cells can express CNGs (Zhang et al. 2002); however, their relevance in the vascular system remains to be determined.

The extent to which cGMP must be elevated to elicit a functional response in the vasculature remains an area of interest. Intracellular cGMP receptors and effectors

may be saturated and functional responses maximal at submaximal cGMP levels. Indeed recent evidence suggests that only very low amounts of cGMP are needed for a full biological response to sGC stimulators in the vasculature (Mergia et al. 2006). Similarly in platelets, a 2–3 fold elevation of intracellular cGMP may be functionally relevant or functionally maximal even if levels can be increased up to 200-fold or more (Gambaryan et al. 2008). Conversely, maximal cGMP efficacy may require maximal cGMP levels and different concentrations of cGMP may be required to observe acute and chronic effects.

1.2 cGMP Cross-Talk and Non-cGMP Signalling

In addition to compartmentalisation there is cross-talk between NO-cGMP and NP-cGMP pathways (see below) as well as between cGMP and cAMP signalling. This includes the capacity for cAMP and cGMP to repress the degradation of its counterpart via actions on phosphodiesterases (PDEs), regulation of cGMP/cAMP synthesis, and cGMP- or cAMP activation of the counterpart protein kinase (cAK and cGK, respectively). Often, however, the elicited actions by cAMP and cGMP are qualitatively similar and both act in a synergistic manner (Pelligrino and Wang 1998). For example, cGMP can either directly cross-activate cAK (Butt et al. 1992), albeit at high concentrations, or indirectly via inhibition of cAMP-specific PDEs (eg PDE3) (Butt et al. 1992; Nolte et al. 1994).

It is important to note that some of the vascular effects of NO and NPs are not necessarily mediated by cGMP. Resistance of NO-mediated responses to the sGC inhibitor, ODQ is indicative of cGMP-independent signalling (Wanstall et al. 2005). NO donor drugs that generate NO "spontaneously", like authentic NO (i.e. solutions of NO gas), appear to exhibit a larger component of cGMP-independent vasorelaxation than do those drugs that require bioactivation in the tissue. In addition, cGMP-independent pathways are most often, though not exclusively, seen at high concentrations (μM - mM) of NO and NO donors. Hence, in relation to the actions of endogenous NO, these pathways may be irrelevant for physiological vasodilation (see also sGC and cGK chapters for genetic knock-out models) but particularly important in settings when the inducible isoform of NOS is expressed. Furthermore, cGMP-independent pathways are enhanced in animal models of atherosclerosis and ischaemia (Wanstall et al. 2005).

Similarly, CNP targets cGMP-independent pathways, hyperpolarising and vasodilating resistance arteries via activation of NPR-C and opening of a G-protein-gated inwardly rectifying K^+ channel (GIRK) (Chauhan et al. 2003; Villar et al. 2007). Furthermore, some of the antiproliferative actions of CNP and ANP may also be mediated via cGMP-independent mechanisms (Cahill and Hassid 1991).

1.3 cGMP Regulates Vasomotor Tone, Cell Growth and Differentiation

1.3.1 Vasomotor Tone

A primary function of cGMP in the vasculature is the regulation of vasomotor tone. Thus NO and NPs (ANP, BNP and CNP) all cause VSM relaxation and in vivo vasodilatation predominantly via cGMP-dependent mechanisms (Moncada and Higgs 2006; Ahluwalia et al. 2004b, Cerra and Pellegrino 2007; Wedel and Garbers 2001). Indeed the importance of endogenous cGMP in the control of vascular tone and blood pressure is highlighted by the observation that eNOS knockout mice are hypertensive (Huang et al. 1995; Shesely et al. 1996) and both ANP and NPR-A knockout mice display chronic and salt sensitive hypertension (John et al. 1995; Melo et al. 1998; Oliver et al. 1997). Global deletion of the cGMP effector, cGKI, however, results in elevated blood pressure at a young age (~32 days) but not in older animals (~43 days) (Pfeifer et al. 1998; Koeppen et al. 2004). Such observations may reflect the development of compensatory mechanisms, an age-dependency of the cGKI signalling pathway or possible cross-activation of cAK by high concentrations of NO that are generated in these aged animals as a consequence of infections and inflammation. Importantly, rescue of global cGKI knockout mice with vascular smooth muscle specific expression of either cGKIα or cGKIβ normalises blood pressure, emphasising the integral role cGK plays in the regulation of vascular homeostasis (Weber et al. 2007).

A substantial body of evidence exists to suggest that vascular tone is regulated in a reciprocal manner by NPs and NO (Hussain et al. 2001; Madhani et al. 2003, 2006; Sabrane et al. 2003). Cross-talk occurs between the NO-sGC and ANP-pGC pathways to regulate cGMP-dependent vasodilatation in vivo in both mice and humans. Thus ANP-induced vasorelaxation (Madhani et al. 2003) and reductions in blood pressure (Madhani et al. 2006) are enhanced in eNOS knockout mice and the hypotensive effects of the NO donor, sodium nitroprusside, are augmented in NPR-A knockout mice (Madhani et al. 2006). Similarly ANP-mediated vasodilatation of the forearm resistance vasculature in man is enhanced following NOS inhibition (Madhani et al. 2006). It is clear that sGC-cGMP and pGC-cGMP systems may serve to compensate for a dysfunction in the other. From a clinical perspective, NP-mediated down-regulation of the NO-cGMP signalling pathway may underlie the observed increased vascular risk in patients with high plasma concentrations of BNP. Conversely, reduced NO-sGC signalling in patients with cardiovascular risk factors and atherosclerosis may be compensated for by increased pGC-derived cGMP.

1.3.2 Cell Growth and Differentiation

Undoubtedly sGC and pGC ligands play an important role in the regulation of endothelial and VSM growth, yet the role of cGMP in mediating these effects is

somewhat controversial (Koyama et al. 2001; Feil et al. 2005). Thus NPs inhibit VSM proliferation (Itoh et al. 1990; Furuya et al. 1991, 1993; Arjona et al. 1997; Yamahara et al. 2003) and enhance endothelial cell regeneration (Doi et al. 2001; Ohno et al. 2002; Kook et al. 2003) in vitro and in vivo, effects which are thought to be mediated predominantly by cGMP. However, some of the anti-proliferative actions of ANP and CNP appear to be cGMP-independent and transduced via activation of NPR-C (Cahill and Hassid 1991). Similarly high concentrations of NO inhibit VSM proliferation (Jeremy et al. 1999; von der Leyen and Dzau 2001; Ignarro et al. 2001), yet the role of cGMP and/or cGKI in these processes is unclear (Feil et al. 2005). In contrast, low concentrations of NO and cGMP have been reported to increase the growth of VSM (Hassid et al. 1994) via stimulation of cGKI (Wolfsgruber et al. 2003). Furthermore, the role of cGMP/cGK in vascular remodelling may also be dependent upon the nature of the remodelling process. Utilising smooth-muscle- specific cGKI deficient mice, cGMP/cGKI was found to promote lesion development in a model of atherosclerosis (Wolfsgruber et al. 2003) but had no impact on vascular remodelling associated with restenosis (Lukowski et al. 2008). These findings further highlight the potential role of cGMP/cGKI independent pathways, such as S-nitrosylation and reactive oxygen species generation, in the anti-remodelling actions of NO (Schleicher and Sessa 2008).

Interestingly, CO, another potential sGC ligand, has antiproliferative effects, suppressing neointimal thickening and vascular stenosis via an increase in cGMP, activation of p38 MAPK and pα 1cip1 (Otterbein et al. 2003). In summary it remains to be determined if the vasculoprotective effects of sGC and pGC ligands are all mediated by cGMP and in which cell type they occur (see below). Nevertheless such anti-mitogenic actions, coupled with the ability to stimulate endothelial cell regeneration, indicate that cGMP elevating agents may slow the progression of diseases such as atherosclerosis and restenosis and potentially offset the vascular hypertrophy and remodelling associated with chronic hypertension.

1.4 cGMP and Endothelial Permeability

cGMP has been implicated in regulating endothelial permeability. While some studies reported a reduced endothelial permeability and antagonistic effects towards cAMP (Hempel et al. 1996), the majority of reports suggest that cGMP increases vascular permeability both in the NO-cGMP (Meyer and Huxley 1992) and NP-cGMP system (Wijeyaratne and Moult 1993). Also permeability increases caused by histamine, bradykinin, serotonin, leukotrienes, platelet activating factor and substance P, all appear to be mediated by cGMP (Meyer and Huxley 1992). Moreover, a consequence of ischemia/reperfusion (IR) is endothelial barrier dysfunction; intravascular volume loss and cGMP generated from endothelial cells appear to mediate this increase in permeability (Ramirez et al. 2006). However there is considerable variability and uncertainty with respect to the appropriate experimental models to be studied. Even the belief that permeability is increased by gaps developing

between the endothelial cells has been challenged (Michel 1998). Thus the final verdict on cGMP's role in vascular/capillary phenomenon is open.

1.5 cGMP and Anti-Inflammatory Actions

cGMP can exert a cytoprotective/anti-thrombotic influence on the blood vessel wall. Thus both sGC- and pGC-derived cGMP have anti-inflammatory activity. NPs prevent immune cell activation, namely ANP prevents iNOS expression in macrophages (Kiemer et al. 2000; Kiemer and Vollmar 1998) and reduces leukocyte infiltration by decreasing the expression of cell adhesion molecules and chemokines (Kiemer et al. 2005). These effects of ANP are largely cGMP-dependent. Similarly, endothelial sGC-derived cGMP (Zabel et al. 2002) appears to play an important role in modulating leukocyte adhesion and rolling (Kubes et al. 1991). Indeed in the vasculature, sGC-derived cGMP (in response to Bay 41-2272) has anti-inflammatory effects, inhibiting expression of the adhesion molecule P-selectin on both endothelial cells and platelets and impairing leukocyte recruitment (Ahluwalia et al. 2004a). Similarly, the anti-platelet activity of NO (Radomski et al. 1987) and NO-independent sGC stimulators (Hobbs and Moncada 2003) and activators (Stasch et al. 2002) is mediated via cGMP (Massberg et al. 1999) and a subsequent down-regulation of P-selectin and glycoprotein IIb/IIIa expression. As such, cGMP elevating agents may have efficacy in the treatment of inflammatory and thrombotic vascular disease.

1.6 Differences in Vascular Regions and Vessel Calibre

Regional and segmental heterogeneity in the vascular responsiveness to cGMP elevating agents has been observed. For example, a greater vasorelaxant potency of the NO donor SNP is apparent in medium-sized versus small intralobar ovine pulmonary arteries (Sathishkumar et al. 2005). However, such an effect is attributable to NO-mediated activation of voltage-dependent K^+ channels (K_V) in the larger vessels rather than a difference in the ability of SNP to elevate cGMP per se (Sathishkumar et al. 2005). Moreover, vascular actions of CNP differ from NPs such that CNP is generally a more efficacious vasodilator of resistance arteries compared with ANP and BNP (Scotland et al. 2005). In addition, CNP exhibits preferential venodilatation (Wei et al. 1993; Banks et al. 1996), whereas ANP has minimal vasorelaxant activity in veins (Wei et al. 1993). Such hetereogeneity is due predominantly to the different vascular distribution of NPR-A and NPR-B receptors (NPR-A expression greater in conduit arteries; Ahluwalia et al. 2004b) and the ability of CNP to target NPR-C in resistance arteries and elicit hyperpolarisation (Chauhan et al. 2003; Scotland et al. 2005; Villar et al. 2007). Other examples include the higher efficacy of sGC activators in diseased (Stasch et al. 2006) and smaller calibre blood vessels (Kemp-Harper et al., unpublished observations).

Differential distribution and expression of PDEs is also likely to play an integral role in conferring heterogeneity to the vascular responses to NO, NPs and PDE inhibitors. Thus the high level of PDE5 in the penile corpus cavernosum and lung vascular smooth muscle (Corbin et al. 2005) selectively targets PDE5 inhibitors to these sites for the treatment of erectile dysfunction (Boolell et al. 1996) and pulmonary hypertension (Cohen et al. 1996), respectively. In addition, an elevation in PDE5 activity in the venous but not arterial vasculature appears to contribute to the development of vascular tolerance to GTN (MacPherson et al. 2006). These are only some examples of regional and calibre-dependent variability in vascular cGMP. We are standing only at the beginning of carefully mapping the relative contribution of different vascular mediators to vascular beds and vessel calibres.

2 cGMP in Vascular Disease

2.1 What Can Go Wrong?

With respect to vascular pathophysiology of cGMP, the initial focus resided on oxidative stress and interference with NO biosynthesis, both by uncoupling the relevant NO synthases (eNOS/NOS3 and, in the adventitia, nNOS/NOS1), accumulation of asymmetric dimethyl-L-arginine, competing with endogenous substrate, L-arginine (Böger 2007), or direct NO scavenging by superoxide. One of the relevant sources of reactive oxygen species appears to be NADPH oxidase (Gavazzi et al. 2006). However, more recent evidence suggests that cGMP generation and signalling can be affected as well. Defects may occur at the level of sGC or beyond and the phosphorylation of VASP can be used as an indicator for defective cGMP-cGK signalling (Ibarra-Alvarado et al. 2002). Thus in SHR rats and hypercholesterolemic rabbits, a decreased sGC expression (Melichar et al. 2004) is observed. In contrast, Jebelovszki et al. (2008) found in obese (fed with 60% of saturated fat) rats unchanged sGC levels and rather an increased sensitivity of sGC to NO. The molecular mechanisms of the latter observations and discrepancies to the rabbit model remain to be elucidated. Nevertheless, sGC is also downregulated in SHR/NDmcr-cp rats, a model of metabolic syndrome (Kagota et al. 2006), but not in pulmonary hypertension (Kirsch et al. 2008) contrary to an earlier report (Wedgwood et al. 2005). Finally, in a lipopolysaccharide-induced model of sepsis, high levels of iNOS/NOS2 derived NO can lead to sub-acute, reversible downregulation of sGC (Fernandes et al. 2006), an observation that fits in vitro observations of sGC downregulation in isolated cells upon prolonged exposure to NO donors (Schmidt et al., unpublished). In addition, using sGC activators as a tool, the occurrence of oxidised/heme-free sGC is enhanced in different disease models and human diabetic vasculopathy (Stasch et al. 2006; Evgenov et al. 2006), leading to augmented sGC degradation (Nedvetsky et al. 2008). The precise mechanism for the higher abundance of oxidised/heme-free sGC under disease conditions is unclear;

while oxidative or nitrosative stress and thiol oxidation are all possibilities, insufficient heme biosynthesis or heme incorporation is also a likely mechanism (Mingone et al. 2006a, b, 2008). Less is known with respect to potential changes in cGKI expression or function in vascular disease or whether the recently described H_2O_2-cGK pathway (Burgoyne et al. 2007) represents a back-up mechanism under oxidative stress conditions when NO-cGMP is impaired (see above). August et al. (2006) have described that basal cGKI activity is decreased in genetically hyperlipidemic rabbits, which may however be primarily due to a reduced sGC expression. Interestingly, Liu et al. (2007) have found a glucose-induced down-regulation of cGKI, mediated by PKC-induced NOX activation and superoxide formation, leading to a decreased NO-induced vasodilation. Thus reduced cGKI levels may contribute to diabetic vasculopathy. Increased PDE activity has been described upon short exposure to NO, causing cross-desensitisation to NPs but not to a direct cGK activator (Müllershausen et al. 2006). Whether neutral endopeptidase (NEP) — the major enzymatic pathway for degradation of NPs, and a secondary for kinins and adrenomedullin — is upregulated under disease conditions is unclear; nevertheless it is emerging as a vascular therapeutic target with vasopeptidase inhibitors employed to inhibit both angiotensin-converting enzyme and NEP in the setting of cardiovascular disease (Rose and Giles 2008; Corti et al. 2001). Similarly the role of the GC-free NPR-C receptor as NP clearance receptor or independent signalling pathway for NPs is open for debate. Finally, tolerance to nitrovasodilators is pathomechanistically irrelevant but a major limitation of current NO-cGMP pharmacotherapy; the recently developed sGC stimulators and activators are devoid of this limitation and likely to supersede nitrovasodilators, making tolerance a less relevant clinical problem.

2.2 Pro- Or Anti-Atherosclerotic, Pro- Antiproliferative

Two areas of cGMP related pathophysiology have seen major dogma shifts, or at least have attempted to do so. One was the suggestion that cGMP could be pro-aggregatory and cause thrombosis (Li et al. 2003); another set of data suggested that cGMP may be proatherosclerotic (Wolfsgruber et al. 2003). Thus all of a sudden a highly protective pathway in the vasculature — at least under acute conditions — had been accused of being potentially detrimental — at least in chronic disease conditions, reviving the old ying-yang hypothesis of cGMP and cAMP (Feil et al. 2005). A thorough investigation of the platelet hypothesis has confirmed the anti-aggregatory function of cGMP (Walter and Gambaryan 2004). The pro- or anti-atherosclerotic role appears to be more complicated. Postnatal smooth-muscle-specific cGKI deletion in the atherosclerosis-prone ApoE-deficient mouse model results in an impaired development of VSMC-derived plaque cells and a significantly decreased lesion area. These results indicated that signalling mechanisms involving endogenous cGKI in VSMCs promote VSMC phenotypic modulation and plaque growth in vivo, and thus a pro-atherosclerotic effect of cGMP

in vivo (Wolfsgruber et al. 2003). However, using a VSMC-specific KO, not all cGK- containing cells may have been appropriately studied. For example, cGK function in inflammatory cells may be important and was unaffected in the VSMC model. Nevertheless at the in vitro level, the VSMC effects match isolated cell studies, where activation of cGKI stimulates the growth of primary VSMCs and a phenotypic switch to more 'atherogenic' cells characterized by increased levels of vascular cell adhesion molecule-1, peroxisome proliferator-activated receptor γ, and phosphatidylinositol-3-kinase–Akt signalling (Wolfsgruber et al. 2003). With respect to NP-cGMP signalling, expression of CNP and its receptors (NPR-B and NPR-C) in human coronary arteries is inversely correlated with the severity of the atherosclerotic lesion (Casco et al. 2002), arguing rather for an overall protective role of cGMP. Also chronic treatment of aged SHR rats with the NO-independent sGC activator, BAY 58-2667, is rather protective on vascular function and morphology (Schmidt et al., unpublished observation). Similarly, chronic treatment with the PDE5 inhibitor, sildenafil, improved NO-dependent vasorelaxation and reduced oxidative stress in aortae from diabetic rats (Schafer et al. 2008). Thus, most likely cGMP is athero-protective when overall effects are analysed, despite the fact that there may be a pro-atherogenic and proliferative component in VSMCs.

3 Therapeutic Applications

3.1 Current Applications

Current vascular applications of cGMP elevating agents include anti-hypertensive therapy (both systemic and pulmonary), coronary artery disease, angina pectoris and atherosclerosis, as well as erectile dysfunction. Most of these are described in other chapters. Currently, anti-hypertensive therapy is limited to nitrovasodilators being given in acute hypertensive crises. Chronic application is prohibited by tolerance development and reflex tachycardia. This indication of sGC stimulation/activation may be considerably extended in future years due to entirely new pharmacological principles. Moreover the potentially potent anti-remodelling effects of cGMP elevating agents may be utilised as powerful anti-atherogenic strategies via decreased leukocyte and platelet activation and adherence and inhibition of smooth muscle proliferation. Finally cGMP elevation is currently being developed as a first line therapy in the treatment of peripheral artery disease (see chapter on sGC activators).

3.2 Potential Applications

In synchrony with the explosive development of the cGMP field in recent years (Kemp-Harper and Feil 2008), several new applications of cGMP elevating or

modulating agents has become apparent and may be targetable with newly developed pharmacological principles and appropriate formulations.

3.2.1 Retinal Blood Vessels

Retinal blood flow is an independent predictor of vascular disease (Wong et al. 2008). Retinal arteries dilate in response to lactate in a cGMP-dependent manner (Hein et al. 2006) and possibly contribute to the metabolic regulation of retinal blood flow. In vitro, the vasomotion frequency but not the amplitude of oscillations of retinal arteries is reduced in response to cGMP or the cGK agonist, 8-Br-cGMP (Hessellund et al. 2006). Clearly, these data show some potential of cGMP modulation in the eye, but also show the need for more preclinical and clinical studies.

3.2.2 Cerebral Blood Vessels, Migraine and Headache

Intracranial arteries of migraineurs are super-sensitive to NO-cGMP (Thomsen 1997) possibly relating to clinical findings suggesting dilatation of the large intracranial arteries on the headache side during spontaneous migraine attacks. Also upon exposure to the NO donor GTN, migraineurs experience a stronger headache than non-migraineurs. Thus migraine may be rare case where a NOS/sGC inhibitor, ideally with cerebral specificity, may be indicated. However, proof-of-principle is lacking.

3.2.3 Peripheral Resistance

Reducing peripheral resistance may be at least as effective with respect to long-term outcome as systemic anti-hypertensive therapy. Mesenteric arteries are often used as an experimental model of resistance-like arteries (Stephens 1987). Slupski et al. (2007) have recently shown that in humans these blood vessels, when isolated under the same conditions as transplanted kidneys, also dilate in a cGMP-dependent manner when using the sGC stimulator, YC-1, although a cGMP-independent effect of this compound (sodium pump stimulation) was observed. Teixeira et al. (2006) had similar findings in rat mesenteric artery using the more specific sGC stimulator, BAY 41-2272. Again some of the effects were medicated by cGMP and endogenous NO; however, high and probably therapeutically irrelevant concentrations of BAY 41-2272 also inhibited Ca^{2+} induced contractions of K^{+} depolarised rings, suggesting interference with Ca^{2+} influx. Importantly, in both species, cGMP is a mediator of vasorelaxation and may be targetable in order to reduce peripheral resistance. Indeed, sGC activators may a first application of this principle by achieving anti-remodelling effects and a reduction in peripheral resistance even at sub-depressor doses (see sGC activator chapter).

3.2.4 Raynaud's Phenomenon

Primary Raynaud's phenomenon is characterized by increased vasoconstrictor tone in the radial arteries that develops during exposure to cold. In women suffering from Raynaud's phenomenon, venous cGMP does not increase in response to cold exposure, which may explain their increased vasospastic response (Leppert et al. 1997). Indeed PDE5 inhibitors appear to be effective in the treatment of Raynaud's disease (Fries et al. 2005; Levien 2006; Rosenkranz et al. 2003).

3.2.5 Placenta

NO-induced relaxation of human placental arteries is partly mediated through cGMP, and partly through a direct effect on potassium channels of the BK(Ca) type (Sand et al. 2006). Whether cGMP elevating agents have any safe therapeutic potential, eg under conditions of placental insufficiency, remains to be seen.

3.2.6 Tolerance

An important component of the anti-anginal efficacy of glyceryl trinitrate (GTN) is attributable to its selective venodilator effect, resulting in decreased cardiac preload and myocardial oxygen demand. Tolerance to GTN develops not only in arteries but also in veins. Indeed cGMP PDE activity is elevated in tolerant veins more so than in tolerant arteries and a PDE inhibitor effectively reverses tolerance in the venous vasculature (MacPherson et al. 2006). Other mechanisms contribute as well. With the development of novel modes of sGC activation or stimulation, other than by classical NO donors (see chapters on sGC stimulators and sGC activators), tolerance as a clinical limitation of chronic cGMP elevating therapy may soon be overcome.

3.2.7 Combination Therapy

Interesting new avenues may lie in the combination of cGMP modulating agents. While the co-administration of NO donors and PDE inhibitors (PDEI) is obsolete, this may not be the case for PDEI and sGC activators or PDEI and ANP (Kemp-Harper and Feil 2008).

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Modulating cGMP to Treat Lung Diseases

Hossein-Ardeschir Ghofrani and Friedrich Grimminger

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Background: Nitric oxide (NO) is constitutively produced in the lung by NO-synthases. The main cellular sources of lung NO production are the vascular endothelium and the airway epithelia (Bohle et al. 2000; German et al. 2000; Ide et al. 1999). Local NO production contributes to regulation of pulmonary perfusion depending on alveolar ventilation to assure optimized ventilation/perfusion distribution (Grimminger et al. 1995). NO-synthase activity is regulated on transcriptional and post-translational redox-based modulation level. The common signaling pathway of endogenous vasodilators, such as nitric oxide, prostaglandins, and natriuretic peptides, engage cyclic nucleotides (cAMP and cGMP). These second messengers are mainly produced by activation of adenylate- and guanylate-cyclases, both membrane-bound and soluble (Beavo 1995). Phosphodiesterases (PDEs) represent a superfamily of enzymes, with PDE1 through PDE11 being currently known, that inactivate cyclic AMP and cyclic GMP, with different tissue distribution and substrate specificities (Ahn et al. 1991; Von Euler and Liljestrand. 1946). Because of stabilization of these second messengers, PDE inhibitors differentially regulate levels of cAMP and/or cGMP, depending on their selectivity profile. Recently, direct

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activators and stimulators of the sGC have been suggested as new therapeutic tools for the treatment of lung vascular disorders that might have even higher potency than PDE inhibitors or exogenously applied NO.

1 Adaptation of Blood Flow to Ventilation in the Pulmonary Circulation

Adaptation of perfusion to ventilation is one of the most important features of pulmonary physiology. The key regulator of this phenomenon is hypoxic vasoconstriction, a reflex originally described by von Euler and Liljestrand (Von Euler and Liljestrand. 1946), which ensures optimized gas exchange (Grimminger et al. 1995; Weissmann et al. 2001). As changes in the distribution of blood flow to different areas of the lung must occur rapidly (e.g., when changing from prone to supine position, or when stressing the pulmonary circulation upon exercise), adjustments of vessel diameter in the respective regions of the lung must be regulated immediately (Cohen et al. 1996; Hillier et al. 1997). A key molecule for this fast response, which links alveolar ventilation (and thus the degree of regional oxygenation) to local lung perfusion, is nitric oxide (NO): it has been conclusively shown that the fall in lung NO production precedes the rise in pulmonary pressure upon induction of acute experimental hypoxic pulmonary vasoconstriction (HPV), as well as that lung NO production is closely related to the degree of alveolar ventilation (Weissmann et al. 2000).

2 Proof of Principle: NO-Inhalation to Improve Gas Exchange in Acute Lung Failure

The adult respiratory distress syndrome (ARDS) is characterized by gas exchange disturbances as a result of (1) loss of alveolar–capillary barrier integrity, with reduced diffusion capacity, (2) increases in ventilation/perfusion mismatch, and (3) acute pulmonary hypertension (Radermacher et al. 1989; Melot et al. 1989). Several attempts have been undertaken to improve gas exchange with systemically applied vasodilators, which resulted in reduction of pulmonary hypertension, but were hampered by concomitant worsening in gas exchange (Rossaint et al. 1993). Inhaled nitric oxide administered in patients with ARDS could for the first time display features of a vasodilator that selectively dilates vessels in the lung (*pulmonary* selectivity), and in addition preferentially dilates vessels in well ventilated areas only (*intrapulmonary* selectivity) (Fig. 1) (Schermuly et al. 2000). Improvements in oxygenation in this patient collective were attributed to a reduction in intrapulmonary shunt-perfusion. In contrast, infused prostacyclin – although potently reducing pulmonary pressure – had no selectivity for the pulmonary circulation (i.e., equipotently reduced systemic vascular resistance) and in addition resulted in deteriorated

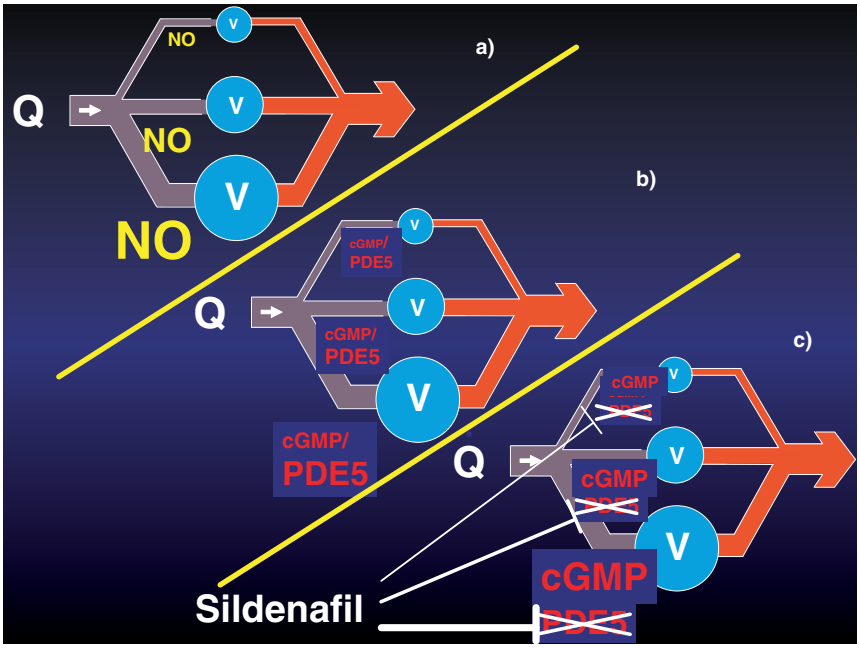

Fig. 1 Rationale for intrapulmonary selective vasodilatation by sildenafil. Nitric oxide (NO) is released in the lung, preferentially in well ventilated areas of the lung (V) and thus directs blood flow (Q) into these regions by local vasodilatation. In contrast, in regions with less ventilation, only little NO is produced and resistance vessels in these regions constrict due to hypoxic pulmonary vasoconstriction reflex (**a**). In areas with high local NO production, more cGMP is released as a second messenger of NO-response and concomitantly PDE5 activity is upregulated. Again only little PDE5 activity is present in areas of less ventilation (**b**). The PDE5 inhibitor sildenafil, despite systemic administration, preferentially acts in areas of good regional ventilation, thereby augmenting the endogenous vasodilatory action of NO redirecting blood flow optimizing ventilation/perfusion matching (**c**)

oxygenation by worsening of ventilation/perfusion matching. However, only few reports support the long term treatment of chronic pulmonary hypertension by continuous therapy with NO inhalation (Channick et al. 1996; Higenbottam et al. 2000).

3 PDE5 in the Pulmonary Circulation: Gateway Opener for Oral Therapy

In patients with idiopathic pulmonary arterial hypertension (iPAH), intravenous prostacyclin has been demonstrated to be a potent pulmonary vasodilator, and long-term infusion of this prostanoid was found to improve exercise tolerance and survival in these patients (Egan 1999). However, in the presence of ventilatory disorders, systemic administration of vasodilators regularly increases the blood flow to low or nonventilated lung areas by interfering with the physiological

hypoxic vasoconstrictor mechanism, thereby worsening preexistent ventilation(V)/perfusion(Q) mismatch and shunt flow (Agusti and Rodriguez-Roisin 1993; Olschewski et al. 1999). Decrease in arterial oxygenation and wasting of the small ventilatory reserve of these patients are the disadvantages of this effect. Inhalation of a vasorelaxant agent to achieve selective pulmonary vasodilation and to redistribute blood flow to the well-ventilated lung areas is an appealing concept to circumvent these hazards (Walmrath et al. 1993; Olschewski et al. 1996). In pulmonary arterial hypertension, daily repetitive aerosolization of the long-acting prostacyclin analogue iloprost has been suggested as a new therapeutic concept (Olschewski et al. 1996, 1998, 2000, 2002; Hoeper et al. 2000). In secondary pulmonary hypertension linked to lung fibrosis, inhaled iloprost was found to decrease the pulmonary vascular resistance similar to intravenous prostacyclin, but not to increase shunt flow as occurred during prostanoid infusion in these patients (Rubin 1997). Continuous inhalation strategies are, however, hampered by practical burdens due to the cumbersome use of inhalation devices as well as the necessity of frequent inhalations over the daytime.

Phosphodiesterases are a superfamily of enzymes that inactivate cyclic adenosine monophosphate and cyclic guanosine monophosphate, the second messengers of prostacyclin and nitric oxide. The phosphodiesterases have different tissue distributions and substrate affinities (Fig. 2); in particular, phosphodiesterase-5 is abundantly expressed in lung tissue (Beavo 1995). The first oral drug to show a selective

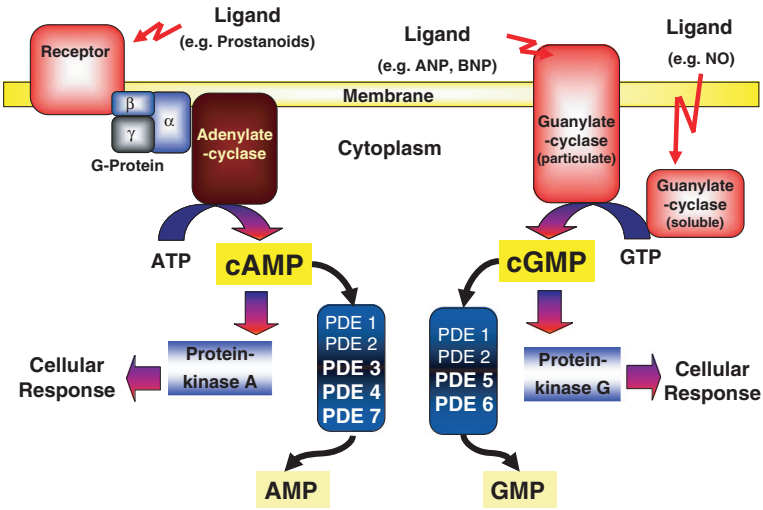

Fig. 2 Scheme of prostanoids, NO, and natriuretic-peptides activated signaling pathways. NO activates soluble- and natriuretic peptides, the membrane-bound guanylate cyclases that synthesize cyclic GMP, which subsequently activates protein kinase G. On the other hand, prostanoids activate the adenylate cyclase, increasing intracellular levels of cAMP with consecutive intracellular signaling. The downstream effects of cGMP and cAMP are limited by phosphodiesterases (PDE), some of which are selectively degrading cAMP or cGMP only, others acting on both mediators

(preferential lung over systemic vasodilation) and "supra-selective" (vasodilation in well-ventilated, but not in nonventilated lung areas) pulmonary vasorelaxation was the phosphodiesterase (PDE) type 5 inhibitor sildenafil: In patients with lung fibrosis and concomitant pulmonary hypertension, known to be susceptible to gas exchange abnormalities due to underlying intrapulmonary shunt flow, the pharmacological properties of sildenafil were tested (Ghofrani et al. 2002b). Oral sildenafil was found to cause pulmonary vasodilation in patients with lung fibrosis and pulmonary hypertension, with an overall potency corresponding to that of intravenous prostacyclin (Fig. 3). Most notably, in contrast to the infused prostanoid, selectivity for well-ventilated lung areas was demonstrated for sildenafil, resulting in an improvement rather than a deterioration of gas exchange (Fig. 4). Controlled randomized trials, however, are needed to confirm the therapeutic benefit in this secondary form of pulmonary vascular disorder. However, this unique profile, never before disclosed for a systemically administered agent, suggested that the PDE-5 inhibitor sildenafil might be a promising candidate for long-term treatment of severe forms of pulmonary hypertension.

4 Clinical Experience with Sildenafil for the Treatment of Chronic Pulmonary Hypertension

A case report of a patient suffering from severe pulmonary arterial hypertension who was treated chronically with very high doses of oral sildenafil indicated that this approach might be effective (Prasad et al. 2000). In pediatric patients, the administration of intravenous prostacyclin is even more hampered by problems associated with i.v. mode of administration than in adults. A study reporting the successful use of oral sildenafil in a child with severe pulmonary hypertension attracted attention, not only within the medical community, but also in the media (Abrams et al. 2000; Patole and Travadi 2002; Oliver and Webb 2002). Trials addressing the characterization of the acute effects of sildenafil on pulmonary and systemic hemodynamics in a larger number of patients with pulmonary arterial hypertension showed that sildenafil effectively reduced pulmonary vascular resistance in a dose-dependent manner (Ghofrani et al. 2002a). In combination with a prostanoid (inhaled iloprost), augmentation of the pulmonary vasodilatory effect of each single agent was observed (Ghofrani et al. 2002a; Wilkens et al. 2001). Long-term treatment of patients with pulmonary arterial hypertension was investigated in a number of single-centre studies, all confirming the efficacy and tolerance of chronic oral sildenafil treatment (Kothari and Duggal 2002; Sastry et al. 2002, 2004). In patients with deteriorating severe PAH despite ongoing prostanoid treatment, additional long-term administration of oral sildenafil improved exercise capacity and pulmonary hemodynamics (Ghofrani et al. 2003a). Thus, the combination of prostanoids and sildenafil has potential as a possible future treatment for pulmonary hypertension. Numerous reports on the clinical use of sildenafil in pulmonary arterial hypertension in uncontrolled trials have been published to date

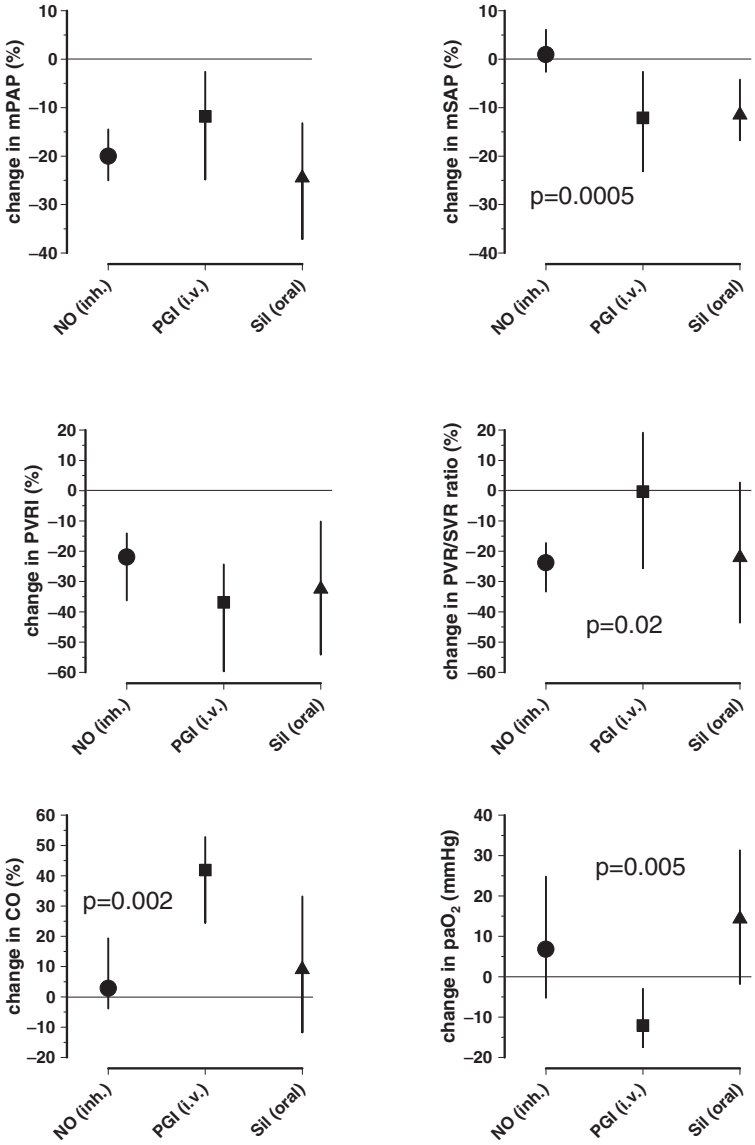

Fig. 3 Hemodynamic and gas exchange response to inhaled NO, infused PGI_2, and oral sildenafil in patients with lung fibrosis and pulmonary hypertension. Deviations from preintervention baseline are displayed for inhaled NO, infused prostacyclin (PGI i.v.), and oral sildenafil (Sil oral). Abbreviations: *mPAP* Mean pulmonary arterial pressure, *mSAP* Mean systemic arterial pressure, *CO* Cardiac output, *PVRI* Pulmonary vascular resistance index, *PVR/SVR ratio* Ratio of pulmonary to systemic vascular resistance, paO_2 Partial pressure of arterial oxygen (changes given in mmHg) (adapted from Ghofrani et al. 2002)

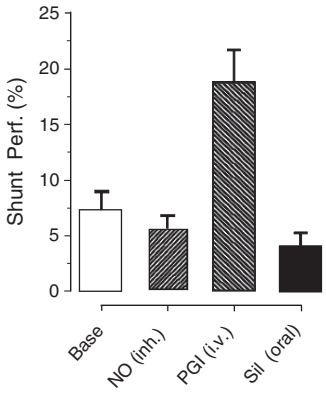

Fig. 4 Pulmonary shunt flow at baseline and in response to vasodilator challenge (assessed by MIGET). The percentage (mean ± SD) of shunt perfusion of total pulmonary blood flow is displayed at baseline (Base), during NO inhalation (NO (inh.)), during infusion of prostacyclin (PGI (i.v.)), and after oral sildenafil (Sil (oral)).

(Kothari and Duggal 2002; Sastry et al. 2002; Watanabe et al. 2002; Zimmermann et al. 2002; Singh et al. 2002; Lepore et al. 2002; Michelakis et al. 2002; Zhao et al. 2003).

Sildenafil appears to be effective for treating patients with pulmonary hypertension of diverse causes in addition to idiopathic pulmonary arterial hypertension. In patients suffering from human immunodeficiency virus (HIV)-related pulmonary hypertension, sildenafil was effective in reducing the pulmonary vascular resistance, as it was in iPAH (Schumacher et al. 2001; Carlsen et al. 2002). Recent data suggest that long-term oral sildenafil treatment in patients with nonoperable chronic thromboembolic pulmonary hypertension or portopulmonary hypertension can be beneficial (Ghofrani et al. 2003b; Reichenberger et al. 2007). The importance of this finding lies in the fact that there is little to offer as a therapeutic option for these patients, with the exception of lung transplantation.

5 Pivotal Trial and Approval of Sildenafil for the Treatment of PAH (SUPER-1)

A growing body of evidence from various studies between 1998 and 2001, which demonstrated the efficacy of sildenafil in the treatment of pulmonary arterial hypertension, led to the design of a large randomized, controlled, multinational trial to provide a final proof of this new treatment concept, and to obtain regulatory approval for sildenafil as a new treatment for pulmonary arterial hypertension. The SUPER-1 (Sildenafil Use in Pulmonary Hypertension) study begun in 2002 and included 278 patients with symptomatic pulmonary arterial hypertension who were treated either

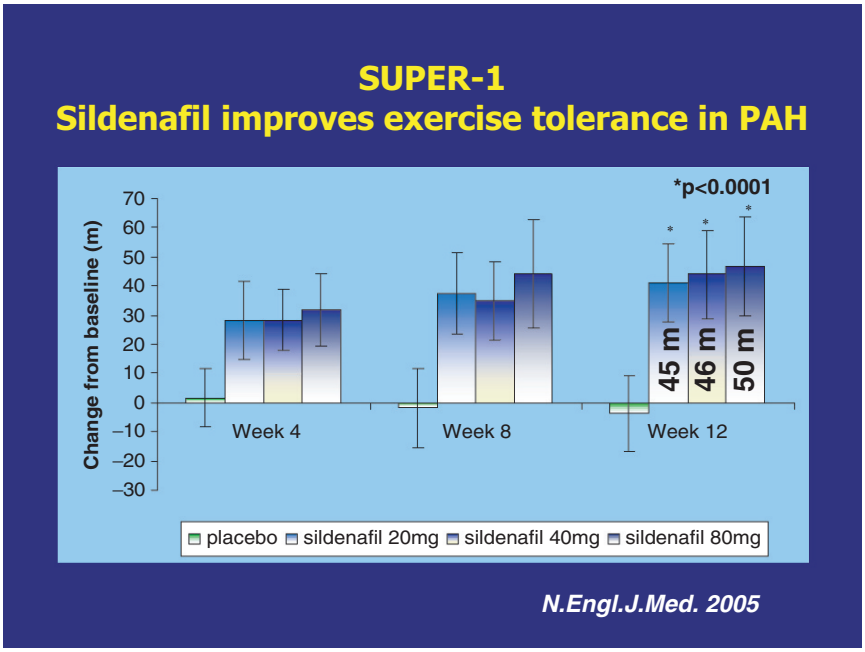

Fig. 5 Key results from the pivotal RCT with sildenafil in PAH (SUPER-1 trial). Changes in the six-minute walking distance as compared to baseline values after 4, 8, and 12 weeks, respectively, are shown in this graph. Patients received either placebo or 20, 40, or 80 mg sildenafil TID

with a placebo or sildenafil (20, 40, or 80 mg) orally, three times daily, for 12 weeks (Fig. 5). The primary end-point of this trial – as in many similar trials with other medications – was a significant improvement comparing baseline to week 12 in the 6-min walk test. Sildenafil (in all the tested doses) improved exercise capacity (up to 50 m (placebo corrected value) in the 80 mg three times daily (TID) group), functional class, and hemodynamics, as compared to placebo-treated patients, and was very well tolerated (Galie et al. 2005). Additionally, patients completing the double-blind phase were able to enter a long-term extension trial, which was conducted over a 2-year period and received 80 mg sildenafil TID. The increase of the 6-min walk distance achieved after 3 months in the placebo-controlled phase was maintained even after 1 year of therapy, as were the improvements in functional class, both results were strongly indicative of the maintenance of the effect despite the severity of the disease. Based on the very favorable mid- and long-term effects of this new oral treatment, sildenafil was approved by the FDA and the EMEA in 2005 for the treatment of patients suffering from PAH. Both agencies decided to approve only the 20 mg TID dose, as only a flat (nonsignificant) dose–effect relationship between 20–80 mg TID was observed regarding the primary end-point of the study, the increase in 6-min walking distance over 12 weeks treatment. An analysis of sildenafil plasma levels in the SUPER-1 study showed no dose–effect relationship with the doses that were studied (data on file, unpublished). There is evidence from some

clinical and experimental settings that the duration of action of sildenafil may not be accurately reflected by plasma-levels and the applied dosage (Moncada et al. 2004). It has been shown that the affinity of sildenafil to PDE5 remains increased after intracellular phosphorylation of the enzyme (Mullershausen et al. 2003). In addition, the conformational changes of PDE5 and the slow dissociation rate of sildenafil from the enzyme may contribute to the flat dose–effect relationship (Francis et al. 1998; Gopal et al. 2001; Corbin and Francis 2002; Huai et al. 2004). Another possible explanation is the hypothesis that sildenafil binding to the catalytic site of PDE5 could occur at higher affinity and may thus retard clearance of the inhibitor from the cell. On the contrary, in the SUPER-1 trial there were clear trends in some secondary endpoints (some showed statistically significant differences between the three applied doses), indicating that, for a subgroup of patients, higher doses might be more efficacious than the approved 20 mg TID dosage. Moreover, in the majority of previous short- and long-term studies, daily doses of 100 up to 300 mg were investigated and reported to be efficacious and well tolerated (Ghofrani et al. 2002a; Wilkens et al. 2001; Kothari and Duggal 2002; Sastry et al. 2004; Michelakis et al. 2003). Thus, future studies are warranted, addressing the long-term efficacy of 20 mg TID or even smaller doses of sildenafil for the treatment of PAH.

6 More PDE5 Inhibitors

In a comparative clinical trial, 60 consecutive PAH patients (NYHA classification II–IV) undergoing right heart catheterization for acute pulmonary vasoreactivity testing received initial short-term nitric oxide inhalation and were subsequently assigned to oral intake of 50 mg sildenafil ($n = 19$), 10 mg ($n = 7$) or 20 mg ($n = 9$) vardenafil, or 20 mg ($n = 9$), 40 mg ($n = 8$), or 60 mg ($n = 8$) tadalafil (Ghofrani et al. 2004). Hemodynamics and gas exchange responses were assessed over a subsequent 120 min observation period. All three PDE-5 inhibitors caused significant pulmonary vasorelaxation, accompanied by an increase of cardiac output, with maximum effects obtained after 40–45 min (vardenafil), 60 min (sildenafil), and 75–90 min (tadalafil). Sildenafil and tadalafil, but not vardenafil, caused a significant reduction of the pulmonary to systemic vascular resistance ratio. Significant improvement in systemic arterial oxygenation, corresponding to that observed during NO inhalation, was noted only with sildenafil. Thus, the three PDE-5 inhibitors appeared to differ in their kinetics of pulmonary vasorelaxation (most rapid effect produced by vardenafil), selectivity for the pulmonary circulation (sildenafil and tadalafil, but not vardenafil), and impact on systemic arterial oxygenation (improvement only after sildenafil). A controlled clinical trial investigating the effects of the PDE5 inhibitor tadalafil in patients with PAH has recently come to an end (LVGY and LVGX); the results will be released in late 2008.

7 Soluble Guanylate Cyclase: A New Target for the Treatment of Pulmonary Vascular Disorders

The downstream effector of NO is the enzyme soluble guanylate cyclase (sGC), which synthesizes the second messenger cyclic guanosine monophosphate (cGMP). Soluble GC is typically found as a heterodimer, consisting of a larger α-subunit and a smaller haem-binding β-subunit. The binding of NO to sGC results in activation and synthesis of the second messenger cGMP. Further, cGMP activates cGMP-dependent protein kinases (PKGs), leading to a reduction of cytosolic Ca^{2+} concentration and desensitization of the actin–myosin contractile system. Although impairment of the endothelium-dependent regulation of pulmonary vascular tone is consistently reported, the analysis of the role of sGC in chronic hypoxia-induced PAH has yielded conflicting data, with both increases and decreases of sGC protein expression described (Hassoun et al. 2004; Li et al. 2001; Li et al. 1999). Therapeutic potential has been reported for YC-1, which acts as an "NO-sensitizer," greatly enhancing the sensitivity of sGC towards NO (Friebe and Koesling 1998; Friebe et al. 1996). YC-1 increases cGMP in smooth muscle cells and induces a dose-dependent vasodilation of endothelium-denuded rat aortic rings (Mulsch et al. 1997; Wegener and Nawrath 1997; Galle et al. 1999). Furthermore, YC-1 has been shown to inhibit the adhesion and aggregation of platelets (Teng et al. 1997; Wu et al. 1995; Friebe et al. 1998). Recently, the compound Bay 41–2272, which stimulates sGC directly and enhances the sensitivity of sGC to NO, was shown to be a systemic and pulmonary vasodilator (Stasch et al. 2001; Boerrigter et al. 2003). Furthermore, it augments the vasodilator response to inhaled NO when acute pulmonary hypertension is produced in lambs (Evgenov et al. 2004). While Bay 41–2272 activates sGC in its native form, another compound, Bay 58–2667, has recently been shown to activate sGC even in its oxidized or heme-free form and independent of the activity of nitric oxide (Stasch et al. 2002). In animal models of pulmonary hypertension, it was demonstrated that both the soluble guanylate cyclase stimulator Bay 41–2272 and the soluble guanylate cyclase activator Bay 58–2667 reverse pulmonary hypertension in chronically hypoxic mice and monocrotaline-injected rats. Notably, treatment with these agents was commenced only after full establishment of pulmonary hypertension and right heart hypertrophy, with structural changes in the lung's vasculature. The compound Bay 41–2272 is a novel NO-independent stimulator of sGC with similar characteristics to YC-1 (but with higher potency of about two to three orders of magnitude) and no PDE 5 inhibitory activity (Straub et al. 2001; Stasch et al. 2002). A deeper molecular understanding and encouraging pre-clinical results with stimulators and activators of sGC warranted further clinical development of compounds from this class for the treatment of pulmonary vascular disorders.

In a first clinical trial of patients with moderate-to-severe PH (pulmonary arterial hypertension, distal chronic thromboembolic PH, or PH with mild to moderate interstitial lung disease), the safety, tolerability, and efficacy of the oral sGC stimulator BAY 63–2521 was evaluated. After the optimal tolerated dose was identified by initial patient studies, pharmacodynamic and pharmacokinetic parameters were assessed following a single dose administration (2.5 or 1 mg) in 19 patients.

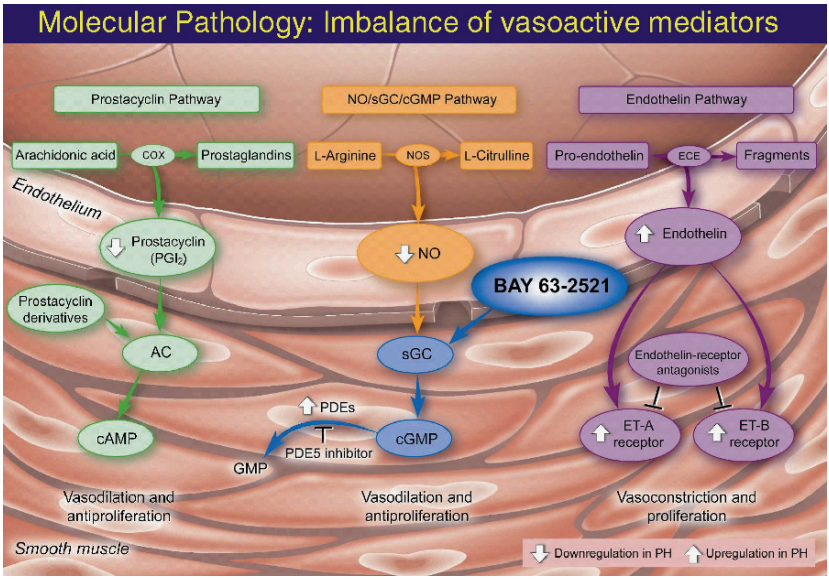

Fig. 6 Molecular pathology of pulmonary hypertension. The three major molecular pathways for pulmonary hypertension that are currently addressed by medical treatments are depicted in this picture. Prostacyclin is produced by prostacyclin synthase and activates the adenylate cyclase (AC), which produces the second messenger cAMP, leading to vasodilatation and antiproliferation. The NO pathway operates via activation of the sGC and elevation of cGMP, resulting in beneficial vasorelaxation and antiproliferation. Therapeutically this pathway can be addressed either by direct stimulation of the sGC, for example, with Bay 63–2521 or by administration of a PDE5 inhibitor. Lastly, the endothelin mediated effects of vasoconstriction and proliferation can be counteracted by the administration of endothelin receptor antagonists

In this short term trial, BAY 63–2521 had a favorable safety profile at a single dose $\leqslant 2.5$ mg. It significantly improved all major hemodynamic parameters in patients with PH in a dose-dependent manner, to a greater extent than inhaled NO, while maintaining mean systolic blood pressure above 110 mmHg. These results supported the view that sGC stimulators may have potential as a novel therapy for PH and warranted further investigation. Recently, clinical phase II and III trials in chronic pulmonary hypertension have been initiated with these compounds. Provided the outcome of these trials is positive, chemical sGC activators and stimulators may soon find their way into the list of molecular targets for the treatment of pulmonary vascular disorders (Fig. 6).

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Modulation of cGMP in Heart Failure: A New Therapeutic Paradigm

Guido Boerrigter, Harald Lapp, and John C. Burnett

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Abstract Heart failure (HF) is a common disease that continues to be associated with high morbidity and mortality warranting novel therapeutic strategies. Cyclic guanosine monophosphate (cGMP) is the second messenger of several important signaling pathways based on distinct guanylate cyclases (GCs) in the cardiovascular system. Both the nitric oxide/soluble GC (NO/sGC) as well as the natriuretic peptide/GC-A (NP/GC-A) systems are disordered in HF, providing a rationale for their therapeutic augmentation. Soluble GC activation with conventional nitrovasodilators has been used for more than a century but is associated with cGMP-independent actions and the development of tolerance, actions which novel NO-independent sGC activators now in clinical development lack. Activation of GC-A by administration of naturally occurring or designer natriuretic peptides is an emerging field, as is the inhibition of enzymes that degrade endogenous NPs.

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Finally, inhibition of cGMP-degrading phosphodiesterases, particularly phosphodiesterase 5 provides an additional strategy to augment cGMP-signaling.

1 Heart Failure

Cardiovascular disease in its various forms is the leading cause of death in the USA. Notwithstanding the different etiologies, a common final stage is the syndrome of heart failure (HF), in which the heart fails to meet the metabolic demands of the body. HF has achieved almost epidemic proportions in terms of increasing prevalence (>5 million in the USA), high incidence (about 550,000 per year), and being the leading cause for hospitalizations (>1 million in 2004) of the elderly (Rosamond et al. 2008). While the widely used New York Heart Association classification provides a measure of current functional status, the new ACC/AHA classification of HF into stages A-D reflects that HF is in most cases a progressive disorder. Stage A represents the presence of risk factors for the development of HF (e.g. hypertension, diabetes mellitus) without structural cardiac disease. In stage B structural cardiac changes (e.g. hypertrophy) are present that are strongly associated with the development of HF. In stage C patients have current or prior symptoms of HF with underlying structural heart disease, and in stage D patients have advanced structural heart disease and refractory HF symptoms at rest (Hunt et al. 2005). With more people surviving into older age, improved treatments for myocardial infarction, and better prevention of sudden cardiac death, the incidence and prevalence of HF is likely to increase further. While pharmacologic (e.g. angiotensin converting enzyme (ACE) inhibitors, beta adrenergic receptor blockers, aldosterone receptor antagonists, nitrates in combination with hydralazine) and device-based (e.g. ventricular assist devices, implantable cardioverter-defibrillators, cardiac resynchronization therapy) treatment modalities have improved patient outcomes, morbidity and mortality remain substantial. Thus, there is a need for novel treatment strategies.

Hallmarks of HF include functional and structural changes in the heart, endothelial and vascular dysfunction with vasoconstriction, sodium and water retention by the kidney, and neurohumoral activation. With regard to new HF treatments, several important points should be noted. HF patients frequently have significant comorbidities and represent an unstable patient population with substantial short-term mortality. Given the heterogeneity of HF, individualized treatment approaches are required. Especially renal dysfunction has emerged as an important determinant of outcome and therapeutic challenge, as in the case of the "cardiorenal syndrome" and diuretic resistance (Liang et al. 2008). Indeed, the requirement to maintain a sufficient renal perfusion pressure is an important limitation to the dosing of vasodilating drugs. In addition, drugs with efficacy in some stages of HF, may be detrimental in others, while improvement in symptoms or hemodynamic function in the short-term may turn out to be harmful in the long term. Also, efficacy of a drug observed when given as monotherapy may be reduced or absent when given on top of standard therapy. The ultimate test for medical interventions that appear rational and promising in preclinical and early clinical studies remains the randomized, controlled clinical trial with appropriate endpoints.

2 Cyclic Guanosine Monophosphate

3′, 5′-cyclic guanosine monophosphate (cGMP) is the second messenger of a variety of signaling systems that use one of several distinct guanylate cyclases (GCs; E.C. 4.6.1.2). GCs are enzymes that convert guanosine 5′-triphosphate (GTP) to cGMP. To date, one cytosolic (soluble) and seven particulate GCs have been identified. Of special importance in the cardiovascular system and illustrated in Fig. 1 are:

- Soluble GC (also called nitric oxide (NO)-sensitive GC) with its endogenous ligand NO

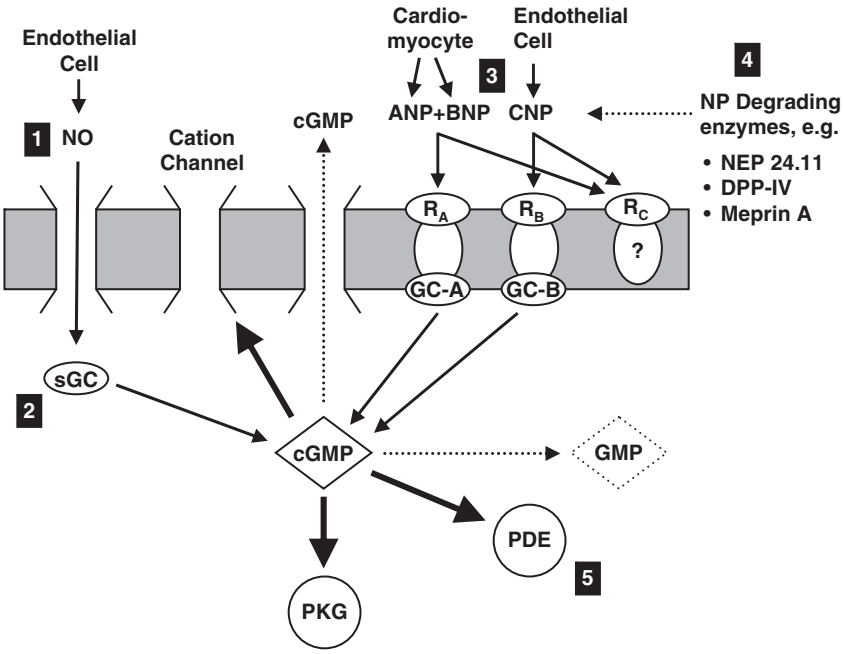

Fig. 1 Simplified schematic of guanylate cyclase (GC) pathways. Cyclic GMP is the second messenger of several distinct signaling pathways. Nitric oxide is produced by endothelial cells and binds to soluble GC in the target cell. ANP and BNP, derived primarily from cardiomyocytes, stimulate GC-A (also called NP receptor A), while CNP, secreted by endothelial cells, stimulates GC-B (also called NP receptor B). Cyclic GMP modulates the activity of cGMP-dependent protein kinase G, cGMP-regulated PDEs, and cGMP-regulated cation channels. The cGMP signal is terminated by a variety of PDEs that hydrolyze cGMP to GMP, or by extrusion into the extracellular space. The NPs are degraded by a variety of peptidases. Cyclic GMP signaling can be augmented by (1) the use of NO-mimetics such as nitrovasodilators, (2) by direct sGC stimulators, (3) by administration of exogenous NPs, (4) by inhibiting NP degrading enzymes, and (5) by inhibiting the activity of cGMP-hydrolyzing PDEs. ANP, atrial natriuretic peptide, BNP, B-type natriuretic peptide, cGMP, cyclic guanosine monophosphate, GMP, guanosine monophosphate, GC, guanylate cyclase, DPP4, dipeptidyl peptidase IV, NEP, neutral endopeptidase, NO, nitric oxide, PDE, phosphodiesterase, PKG, protein kinase G, R_A, natriuretic peptide receptor A, sGC, soluble guanylate cyclase

- GC-A (also called natriuretic peptide A receptor (NPR-A)) with its endogenous ligands ANP and BNP
- GC-B (also called NPR-B) with its endogenous ligand CNP.

Other GCs are GC-C (ligands: guanylin and uroguanylin, heat-stable enterotoxins) and GC-D, -E, -F, and -G for which the endogenous ligands still need to be identified.

Cyclic GMP affects the activity of a variety of effector molecules such as cGMP-dependent protein kinase (PKG), cGMP-regulated PDEs, and cGMP-regulated cation channels. Conversely, cGMP itself is a substrate of several PDEs, which hydrolyze cGMP to GMP and thus terminate the cGMP signal (Bender and Beavo 2006; Conti and Beavo 2007). Of note, by modulating PDEs which catabolize cAMP, cGMP can affect cAMP based signaling systems, such as catecholamines (Zaccolo and Movsesian 2007).

Despite having a common second messenger, activating different cGMP-dependent pathways will not necessarily result in similar actions. Receptors can differ both in their tissue and their intracellular distribution. Within the cell it has been shown that GC-A stimulation as compared to sGC stimulation increases distinct cGMP pools that are compartmentalized by the activity of PDEs (Castro et al. 2006; Piggott et al. 2006; Fischmeister et al. 2006; Takimoto et al. 2007; Hart et al. 2001). These findings are consistent with the observation of distinct actions profiles: e.g. ANP, BNP and NO have vasodilating actions, but only ANP and BNP are natriuretic. Regarding renal function, intrarenal administration of the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) in experimental acute HF reduced renal blood flow without affecting GFR and sodium excretion, whereas natriuretic peptide receptor blockade with HS-142-1 did not affect renal blood flow but decreased GFR and sodium excretion (Martin et al. 2007). It should also be noted that there can be reciprocal regulation of different cGMP pathways; e.g. in vascular smooth muscle cells activation of GC-A leads to an attenuated response to sGC and vice versa (Hussain et al. 2001; Madhani et al. 2006).

The remainder of this review will discuss specific strategies to augment cGMP signaling pathways in HF.

3 The NO-sGC/cGMP Pathway

Soluble GC is a heterodimeric heme enzyme, consisting of an α- and β-subunit and a prosthetic heme group with a ferrous iron. Binding of NO, which is produced by one of three nitric oxide synthases, to the heme iron changes the conformation of the enzyme and increases its catalytic activity dramatically. Signaling via the NO/sGC/cGMP pathway has been shown to be attenuated in a variety of cardiovascular disease states. Part of this is due to scavenging of NO preventing activation of sGC. Indeed, the HF syndrome is associated with markedly increased oxidative stress in vascular and renal tissues contributing to endothelial dysfunction, which is adversely associated with patient outcome. More recent studies have demonstrated that not only can NO be scavenged but also the heme moiety of sGC can be oxidized or missing, rendering the enzyme insensitive to NO (Stasch et al. 2006; Munzel et al. 2007). This endothelial or vascular dysfunction provides a rationale for the therapeutic enhancement of the NO/sGC/cGMP pathway.

3.1 Conventional Nitrovasodilators

Soluble GC stimulation with nitrovasodilators has been employed for more than a century in cardiovascular disease (Brunton 1867). However, long-term administration of nitrovasodilators is associated with the development of tolerance and cGMP-independent actions, which may have beneficial but also detrimental actions. The latter include oxidative stress, mitochondrial toxicity, endothelial dysfunction, and protein nitrosation (Munzel et al. 1995; Caramori et al. 1998; Warnholtz et al. 2002; Sydow et al. 2004; Hess et al. 2005; Heck et al. 2005; Stasch et al. 2006; Thomas et al. 2007). These side effects can at least in part be prevented by hydralazine, which has potent antioxidant properties. Indeed, clinical trials in which patients received a combination of a nitrate (isosorbide dinitrate) and hydralazine demonstrated improved outcomes compared to placebo before the introduction of ACE inhibitors (Cohn et al. 1986) and more recently in African Americans even on top of therapy with ACE inhibitors and beta blockers (Taylor et al. 2004).

3.2 NO-Independent Stimulators and Activators of sGC

Ko et al. (1994) reported that the molecule YC-1 activates sGC directly, i.e. independently of NO, which stimulated the search for similar compounds. Stasch et al. (2001, 2002) described two new classes of direct sGC stimulators, one NO-independent but heme-dependent, the other NO- and heme-independent. The former class only stimulates sGC if the heme iron is in the ferrous state (Fe^{2+}), whereas the latter class activates the enzyme preferentially if the heme iron is oxidized (Fe^{3+}) or the heme is absent.

3.2.1 NO-Independent but Heme-Dependent sGC Stimulators

The first orally available NO-independent but heme dependent sGC stimulator to be described was BAY 41–2272, a pyrazolopyridine. It binds to a regulatory site on the alpha subunit of sGC and activates sGC synergistically with NO and can thus be considered a “NO sensitizer”. In experimental studies BAY 41–2272 lowered blood pressure, relaxed blood vessels that had been made tolerant to nitrovasodilators, and inhibited platelet aggregation. A related compound, BAY 41–8543, prolonged binding of NO to sGC 220-fold (Schmidt et al. 2003). In a canine model of HF induced by rapid right ventricular pacing, BAY 41–2272 decreased mean arterial pressure, pulmonary capillary wedge pressure and systemic and renal vascular resistances and increased cardiac output and renal blood flow (Boerrigter et al. 2003). The hemodynamic actions were similar to nitroglycerin with the important exception of right atrial pressure, which decreased with nitroglycerin but was unchanged with BAY 41–2272. This difference could be due to the fact that BAY 41–2272 acts synergistically with NO, which may be more abundant in arteries than veins. In contrast, nitroglycerin is bioactivated preferentially in the veins. An at least theoretical prob-

lem with the synergism is the potential for a "steal phenomenon", i.e. BAY 41–2272 may be most efficacious in areas with higher endogenous levels of NO, thus potentially diverting blood away from diseased and ischemic areas with low endogenous NO. Also, venodilation, indicated by the decrease in right atrial pressure seen with nitroglycerin, would be desirable in most patients with HF, as it reduces preload and chamber dimensions, and thus wall stress and myocardial oxygen demand. It should be noted that actions of BAY 41–2272 other than stimulating sGC have been discussed, e.g. inhibition of PDE5 (Mullershausen et al. 2004; Bischoff and Stasch 2004). While this would make BAY 41–2272 a less specific drug, it would not necessarily make it a worse therapeutic as is discussed in more detail later in this review.

The NO-independent but heme-dependent sGC stimulator in the most advanced stage of clinical development is BAY 63–2521 (riociguat), which is currently in clinical trials for the treatment of pulmonary arterial hypertension (Ghofrani et al. 2007). Other NO-independent but heme-dependent sGC stimulators are CFM-1571 and A-350619 (Selwood et al. 2001; Miller et al. 2003; Evgenov et al. 2006).

3.2.2 NO- and Heme-Independent sGC Activators

The first reported NO- and heme-independent sGC activator was BAY 58–2667, an amino dicarboxylic acid that binds to regulatory sites on the α- and β-subunit of sGC (Stasch et al. 2002). BAY 58–2667 potently activates sGC because it can compete with and replace the heme moiety, which in the non-nitrosylated state inhibits enzyme activity (Schmidt et al. 2004). Indeed, BAY 58–2667 activates preferentially the oxidized and heme-free enzyme, which makes BAY 58–2667 an interesting pharmacologic tool. Stasch et al. (2006) reported that in various experimental cardiovascular disease conditions the potency of nitrovasodilators was reduced, whereas it was increased for BAY 58–2667, strongly suggesting an increased prevalence of oxidized or heme-free sGC. These findings provide evidence for a potential additional pathophysiologic mechanism contributing to endothelial and vascular smooth muscle cell dysfunction in cardiovascular disease states. They also suggest that BAY 58–2667 may induce the opposite of a "steal phenomenon" by being most efficacious in diseased vessels.

In vivo, BAY 58–2667 has an additive effect with NO and lowered blood pressure in rats and healthy canines (Stasch et al. 2002). In experimental pacing-induced HF, BAY 58–2667 had hemodynamic actions similar to nitroglycerin with reductions in mean arterial and pulmonary capillary wedge pressure, systemic and renal vascular resistance, and increases in cardiac output and renal blood flow (Boerrigter et al. 2007a) (Fig. 2). Importantly, unlike BAY 41–2272, BAY 58–2667 also decreased right atrial pressure. GFR and urinary sodium excretion were preserved despite the hypotensive actions. There was also no activation of the renin-angiotensin-aldosterone system, whereas ANP and BNP decreased, consistent with cardiac unloading.

As BAY 58–2667 possesses impressive hemodynamic properties in experimental HF and preserves renal function, an unblinded, uncontrolled proof-of-concept study was recently undertaken and completed. Specifically, Lapp et al. (2008) assessed

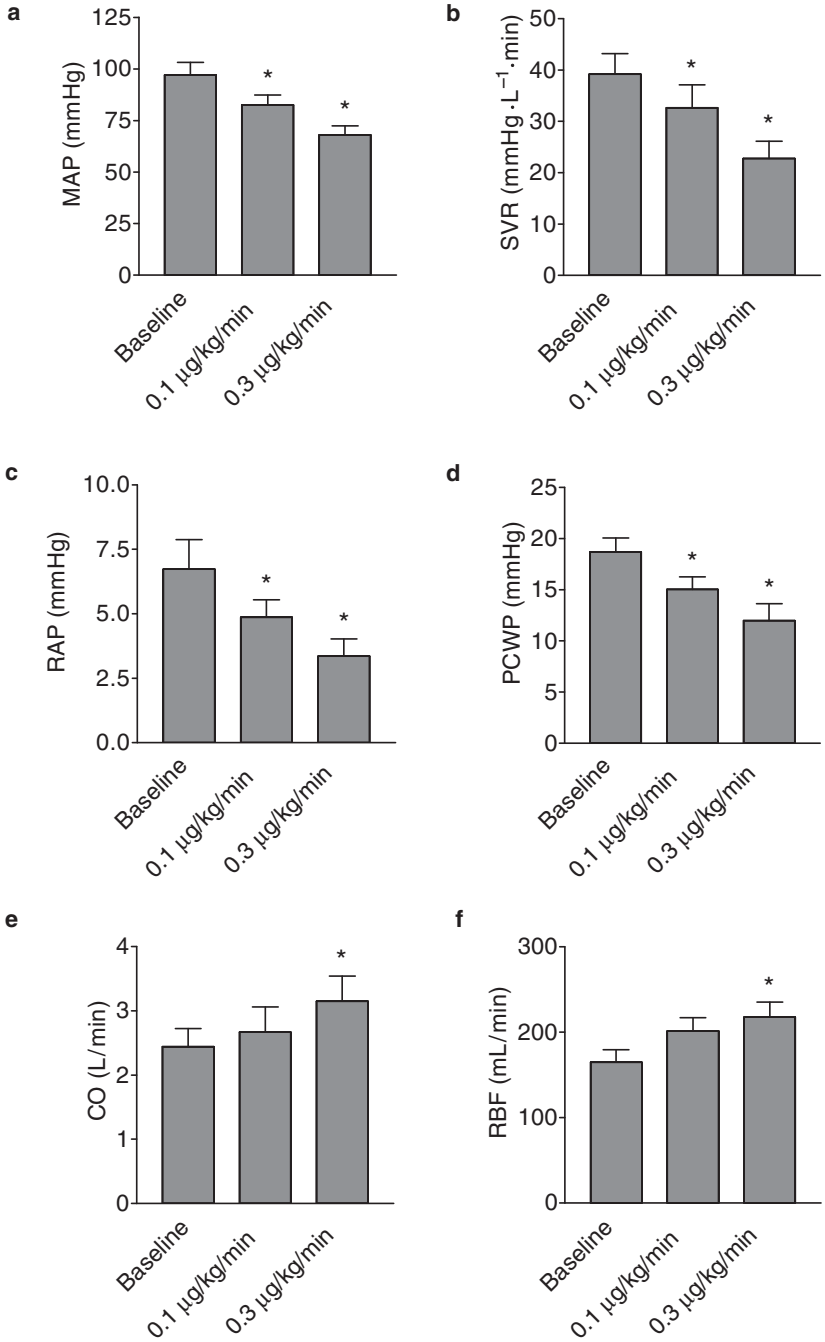

Fig. 2 Effect of BAY 58–2667 administration on **a** mean arterial pressure (MAP), **b** systemic vascular resistance, **c** right atrial pressure (RAP), **d** pulmonary capillary wedge pressure (PCWP), **e** cardiac output (CO), and **f** renal blood flow (RBF). * indicates $p < 0.05$ compared to baseline. Taken from Boerrigter et al. (2007a)

the effect of BAY 58–2667 on hemodynamic and symptomatic status in patients admitted to the hospital with acute decompensated HF, NYHA class III/IV symptoms, PCWP⩾18 mmHg, parenteral pharmacotherapy, and invasive hemodynamic monitoring. Based upon an initial dose-finding part of the study (n = 17), a starting dose of 100 μg h^{-1} was chosen for the proof-of-concept second part of the study (n = 33), in which BAY 58–2667 was infused for a total of six hours. Hemodynamic function was assessed at baseline, after 2, 4, and 6 h of drug infusion, and 2 h after the end of infusion. Dose adjustments could be made at 2 and 4 h; the maximal dose of 400 μg h^{-1} was administered to sixteen of 30 patients who completed the study. BAY 58–2667 significantly reduced PCWP, right atrial pressure, systemic and pulmonary vascular resistance, while cardiac output increased. A PCWP reduction of ⩾4 mmHg was achieved in 53, 83, and 90% after 2, 4, and 6 h, respectively, which was associated with an improvement in symptomatic status as assessed by a dyspnea score. This study shows that also in human HF there exists a pool of sGC that can be activated by BAY 58–2667 with subsequent favorable cardiac pre- and afterload reduction, warranting further studies. Other NO- and heme-independent sGC activators are HMR1766 (ataciguat) and S3448 (Schindler et al. 2006).

When these preclinical and clinical studies are taken together, the results are encouraging and provide evidence for therapeutic potential. As recently stated by Munzel et al. (2007), "these agents could revolutionize the treatment of not only HF but also other conditions, such as coronary artery disease, systemic hypertension, and pulmonary hypertension."

4 The Natriuretic Peptide Pathways

The natriuretic peptides ANP and BNP are secreted by the heart in conditions of cardiac overload and via GC-A stimulate the production of cGMP. They have natriuretic, diuretic, vasodilating, renin- and aldosterone suppressing actions that help to unload the heart. In addition, they have antihypertrophic and antifibrotic properties (Tsuruda et al. 2002; Holtwick et al. 2003) as well as lusitropic (Lainchbury et al. 2000). CNP of endothelial origin functions via activation of GC-B and serves as a more local autocrine/paracrine peptide in endothelial cell/vascular smooth muscle cell control (Stingo et al. 1992). As discussed above, the intracellular cGMP signal of the NPs is terminated by PDEs.

NPs are inactivated in several ways: enzymatic degradation, endocytosis after binding to the natriuretic peptide clearance receptor (NPR-C), or renal excretion. Of note, ANP, BNP, and CNP bind to NPR-C, the main function of which was initially believed to be clearance of NPs; however, other functions have emerged (Villar et al. 2007; Huntley et al. 2006). Importantly, in more advanced HF stages the response to endogenous and exogenous NPs is blunted, which can at least partially be overcome by administration of exogenous NPs, providing a rationale for pharmacologically augmenting the NP system. This is possible in various ways, e.g. administration of exogenous NPs or prolonging the bioactivity of endogenous NPs by inhibiting their degradation.

4.1 Guanylate Cyclase Activators

4.1.1 B-Type Natriuretic Peptide

BNP has been reported to have vasodilating, natriuretic, diuretic, lusitropic, antihypertrophic, and antifibrotic properties, all of which could be beneficial in HF (Yoshimura et al. 1991; Lainchbury et al. 2000; Tsuruda et al. 2002; Tamura et al. 2000). The response to exogenous BNP is blunted, suggesting resistance to BNP and providing a rationale for exogenous supplementation despite high endogenous levels. In canine tachypacing-induced HF BNP augmented the diuretic and natriuretic response to furosemide, increased GFR, and prevented an increase in aldosterone induced by furosemide alone (Cataliotti et al. 2004).

Nesiritide, a recombinant form of human BNP, was approved for the treatment of acute decompensated HF in the US in 2001. In the VMAC trial, nesiritide was administered as a bolus ($2\,\mu g\ kg^{-1}$) followed by a continuous infusion ($0.01-0.03\,\mu g\ kg^{-1}\ min^{-1}$) in patients with acute decompensated HF; in a subset of patients the dose could further be increased in steps of $0.005\,\mu g\ kg^{-1}\ min^{-1}$ preceded by a $1\,\mu g\ kg^{-1}$ bolus up to a maximum of $0.03\,\mu g\ kg^{-1}\ min^{-1}$ (VMAC-Investigators 2002). Nesiritide led to a larger decrease in pulmonary capillary wedge pressure at 3 and 24 h as compared to placebo and nitroglycerin, although symptomatic status was not improved compared to nitroglycerin. Subsequent meta-analyzes of publicly available studies suggested that nesiritide was associated with an increase in serum creatinine and mortality (Sackner-Bernstein et al. 2005a, b). The mechanism for these findings is unclear but possibly the nesiritide dose administered, particularly the bolus, may have contributed to hypotension, which due to BNP's half-life would be more prolonged as compared to e.g., nitroglycerin. Subsequent small studies reported no indication for detrimental or beneficial renal effects of nesiritide in acute decompensated HF (Wang et al. 2004; Witteles et al. 2007; Owan et al. 2008; Burnett and Korinek 2008). Of note, despite the fact that administration of a bolus was left at the discretion of the physician in the latter study, patients with nesiritide had significantly lower blood pressures, which may have offset some potentially beneficial actions by reducing renal perfusion pressure. The manufacturer-sponsored ongoing "Acute Study of Clinical Effectiveness of Nesiritide in Decompensated Heart Failure" (ASCEND-HF) with rehospitalization due to HF and all-cause mortality as a primary outcome measure may be able to provide definitive clarification. Using an innovative but more invasive approach, intrarenal administration of nesiritide with a bifurcated renal catheter in experimental canine HF resulted in a larger natriuresis and increase in glomerular filtration rate as compared to systemic nesiritide (Chen et al. 2006b). Importantly, intrarenal unlike systemic administration did not lower systemic arterial blood pressure.

Nesiritide has also been evaluated in settings other than acute decompensated HF. The FUSION-II trial evaluated once or twice weekly outpatient infusions of nesiritide for 12 weeks in patients with chronic HF on standard therapy. No increased renal dysfunction or mortality, but also no benefit was observed (Cleland et al. 2007). One reason for the lack of efficacy could be that only intermittent

administration of nesiritide was used. Feasibility of chronic subcutaneous administration has been demonstrated in animals and in humans (Chen et al. 2000, 2004) and studies to further explore the efficacy of chronic nesiritide are ongoing. In addition, peptide modification to increase the half-life of BNP or to allow oral delivery have been reported (Cataliotti et al. 2005).

In the NAPA trial, nesiritide (0.01 μg kg^{-1} min^{-1}; no bolus; n = 141) or placebo (n = 138) was given to patients with a left ventricular ejection fraction ⩽40% undergoing bypass surgery with anticipated cardiopulmonary bypass (Mentzer et al. 2007) (Fig. 3). Patients randomized to nesiritide had a smaller rise in serum creatinine, larger urine output, shorter hospital stay, and lower 180-day mortality. Similarly, in a study of patients with reduced renal function undergoing cardiac bypass surgery randomization to nesiritide as compared to control improved postoperative renal function (Chen et al. 2007). Another possible indication where studies are pursued is nesiritide as a treatment to ameliorate remodeling post myocardial infarction.

4.1.2 Atrial Natriuretic Peptide

ANP (carperitide) has been approved for the treatment of HF in Japan since 1995 (Suwa et al. 2005). However, no double-blind placebo controlled studies are available evaluating its efficacy in HF. Most recently, in patients with acute myocardial infarction, carperitide as an adjunct to reperfusion therapy reduced infarct size as estimated by creatine kinase and increased left ventricular ejection fraction (Kitakaze et al. 2007). Similarly, in a study in patients with first anterior myocardial infarction, carperitide decreased left ventricular end diastolic volume and increased ejection fraction compared to infusion of isosorbide dinitrate (Kasama et al. 2007).

Urodilatin (ularitide) is a splice-variant of the ANP precursor with four additional N-terminal amino acids. It is secreted into the urine and does not appear in the circulation. In a phase II trial, 24-hour infusion of ularitide in patients with decompensated HF (n = 221) dose-dependently decreased cardiac filling pressures and systemic arterial pressure and improved symptomatic status with no adverse effects on renal function observed during the first three days after study drug administration (Mitrovic et al. 2006). The most common adverse effect was hypotension, which required at least temporary interruption of drug administration in 5.0, 9.4, and 12.7% in the groups randomized to 7.5, 15, and 30 ng kg^{-1} min^{-1} of ularitide, respectively.

4.1.3 Dendroaspis Natriuretic Peptide

There are also other sources of naturally occurring NPs. Snake venom has been the source of several peptides that have been useful in the development of cardiovascular drugs, e.g. the ACE inhibitor captopril and the glycoprotein IIb/IIIa antagonist eptifibatide. In 1992, Schweitz et al. reported the in vitro biological actions of a newly discovered peptide *Dendroaspis* natriuretic peptide (DNP), which was isolated from

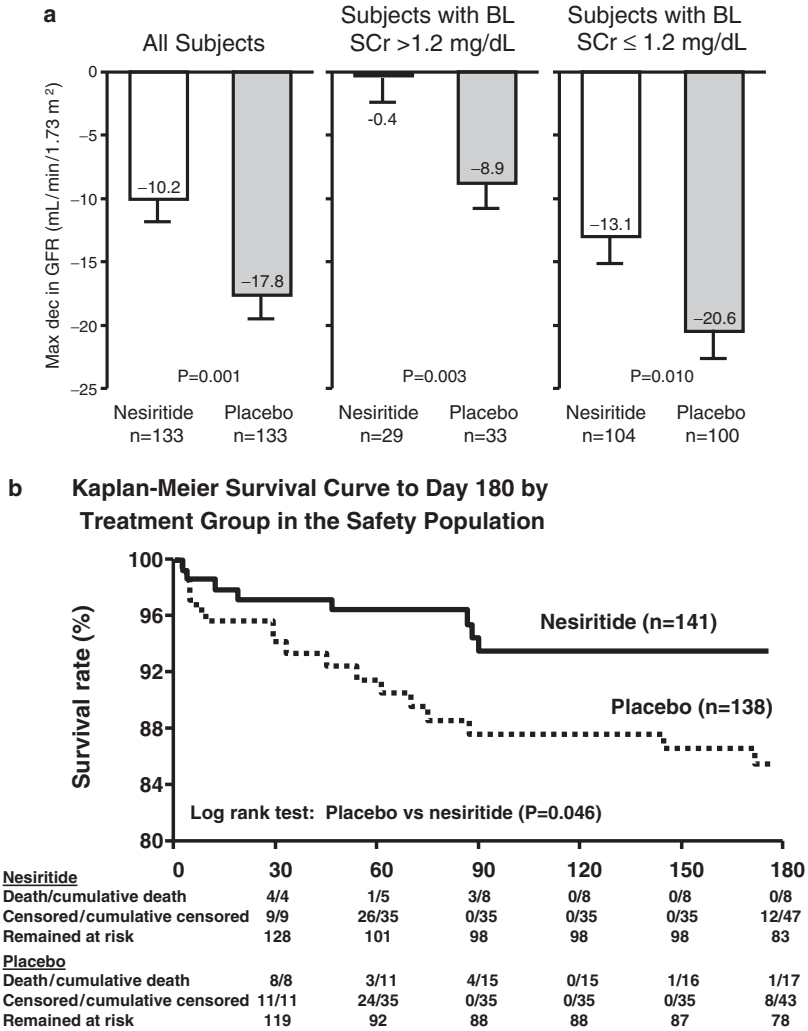

Fig. 3 Adjusted mean maximum decrease in glomerular filtration rate (GFR) from baseline through hospital discharge or by study day 14, whichever came first, using an analysis of covariance model. Error bars indicate standard error of the mean. BL SCr denotes baseline serum creatinine **(a)**. Kaplan–Meier survival curve to day 180 by treatment group in the 'safety population', which is a subset of the total study population added during the study to assess long-term safety **(b)**. Taken from Mentzer et al. (2007); reprinted with permission from Elsevier

the venom of the green mamba. DNP binds to GC-A and the natriuretic peptide clearance receptor, but not to GC-B (Schweitz et al. 1992; Johns et al. 2007). We reported that DNP in vivo was potently natriuretic and diuretic and possessed cardiac unloading actions but with significant hypotensive properties (Lisy et al. 1999, 2001). These in vivo actions of DNP are consistent with GC-A activation as such effects closely mimic the properties of ANP and BNP but not CNP. Of note, DNP

has a higher affinity for the GC-A receptor as compared to ANP and BNP (Singh et al. 2006; Johns et al. 2007). As a non-endogenous peptide, there is however the potential of immunogenicity.

4.2 Guanylate Cyclase B Activators: C-Type Natriuretic Peptide

Another member of the endogenous NP family is CNP, which acts via GC-B (Tawaragi et al. 1991). CNP is a 22-amino acid (AA) peptide that shares structural homology with the other members of the NP family, all of which function via well-characterized particulate GC receptors as discussed above and the second messenger cGMP. While possessing structural similarity, CNP is genetically distinct from ANP and BNP. Also, unlike ANP or BNP, CNP lacks a carboxy-terminal amino acid extension, which may explain in part its lack of natriuretic properties (Clavell et al. 1993; Hunt et al. 1994). Since its discovery in 1990, we have learned that CNP is principally an endothelial cell derived peptide (Stingo et al. 1992). In isolated venous and arterial rings, CNP activates GC-B receptors in veins while ANP and BNP activate GC-A receptors in both arteries and veins which is consistent with the less hypotensive actions of CNP as compared to ANP and BNP (Wei et al. 1993; Igaki et al. 1998; La Villa et al. 1998).

Studies have also demonstrated that CNP possesses more potent anti-proliferative and collagen suppressing properties in cardiac fibroblasts as compared to ANP and BNP (Horio et al. 2003). Relevant to such antifibrotic properties, studies have reported that 14 days of continuous infusion of CNP in rodents with acute myocardial infarction (AMI) markedly attenuates ventricular dilation, cardiac fibrosis, and cardiomyocyte hypertrophy (Soeki et al. 2005). The chronic infusion of CNP was without any hypotensive actions.

In clear contrast to ANP and BNP, CNP lacks significant natriuretic and diuretic actions when infused into humans. This may explain its lack of utility in sodium and water retaining syndromes such as HF despite its attractive venodilating and antifibrotic properties. Interestingly, in HF GC-A is down regulated and GC-B is relatively more abundant than GC-A (Dickey et al. 2007; Bryan et al. 2007) which provides a rationale for GC-B agonist-based therapies in HF.

4.3 Designer Natriuretic Peptides

Given the complexity and diversity of HF, it should come as no surprise that currently available NPs are not efficacious or ideal for every patient. Indeed, as discussed in the section on BNP (4.1.1) hypotensive actions or specific renal resistance in some patients may completely offset any renal enhancing actions. Thus, attempts are made to design and develop NPs with an improved profile of action. Aims of

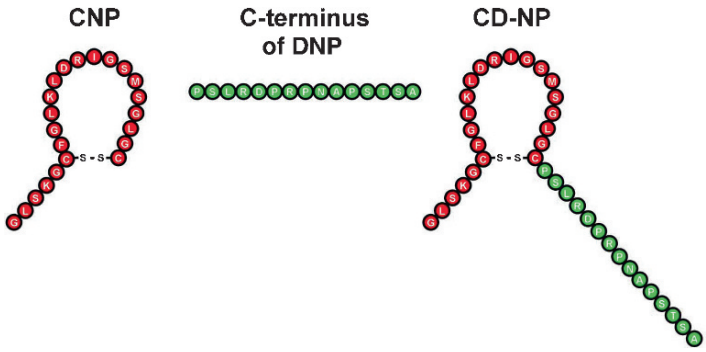

Fig. 4 CD-NP is a chimeric peptide consisting of the amino terminus and ring structure of C-type natriuretic peptide and the carboxy terminus of Dendroaspis natriuretic peptide

modifying the peptide sequence could be to change the affinity to different receptors and the susceptibility to enzymatic degradation.

A key structural feature of DNP is that it possesses the longest C-terminus of the known natriuretic peptides consisting of 15 amino acids (AA) as compared to 5 AA for ANP, 6 AA for BNP, and none for CNP. Indeed, the long C-terminus of DNP may render DNP highly resistant to degradation by neprilysin (NEP; EC 3.4.24.11; also called neutral endopeptidase, CD10) contributing to potent natriuretic and diuretic actions (Chen et al. 2000). Further, the lack of a C-terminus for CNP may explain the observation that of the three known endogenous natriuretic peptides CNP is the most susceptible to degradation by NEP, which could limit its renal actions as NEP is strongly expressed in the kidney (Kenny et al. 1988).

Based upon exploratory studies of the 15-AA C-terminus of DNP, we found that fusion of the 15 AA C-terminus of DNP into the C-terminus position of the core 22-AA ring structure of CNP resulted in a synthetic chimeric peptide that in vivo possessed the cardiac unloading actions of CNP with minimal hypotensive properties together with the additional renal effects of natriuresis and diuresis (Lisy et al. 2008) (Fig. 4). We also observed that this chimera called CD-NP retained properties of CNP in vitro in activating cGMP in cardiac fibroblasts and inhibiting cell proliferation. Thus, this chimeric natriuretic peptide possesses potentially beneficial efficacy and safety for the treatment of cardiorenal disease states such as HF and AMI. A first in human study was recently completed in normal human subjects and demonstrated activation of plasma and urinary cGMP, natriuresis, aldosterone suppression with minimal actions on arterial pressure (Lee et al. 2008).

4.4 Inhibitors of Natriuretic Peptide Degradation

An alternative way to augment the NP pathways is to inhibit enzymes involved in the degradation of endogenous NPs. However, as these enzymes are likely to have other substrates, this approach is a less specific strategy and a potential advantage

of augmenting the NPs may be offset by reduced degradation of undesired endogenous peptides. Enzymes reported to be involved in NP degradation are NEP, dipeptidyl peptidase IV (DPP-IV; EC 3.4.14.5; also called CD26), and meprin A (EC 3.4.14) (Kenny and Stephenson 1988; Brandt et al. 2006;Pankow et al. 2007). NEP inhibitors have been tested in clinical trials but were not superior to placebo (Northridge et al. 1999; Cleland and Swedberg 1998). Omapatrilat is a vasopeptidase inhibitor that simultaneously inhibits ACE and NEP. In the Omapatrilat Versus Enalapril Randomized Trial of Utility in Reducing Events (OVERTURE, n = 5770), omapatrilat demonstrated equal efficacy as enalapril regarding death or hospitalization for HF (Packer et al. 2002). Interestingly, had the end point criteria of the Studies of Left Ventricular Dysfunction (SOLVD) trial been employed, a statistically significant improvement with omapatrilat would have been found. However, in an echocardiographic OVERTURE substudy, no differences in LV remodeling and function was observed between randomization groups (Solomon et al. 2005). Of note, given that omapatrilat is a more potent and longer lasting inhibitor of ACE as compared to NEP, the once daily administration of omapatrilat may not have been sufficient to persistently enhance the NP system and demonstrate superiority as compared to ACE inhibition alone in this HF study population. In the Exercise and Symptoms Study of HF(IMPRESS, n = 573), omapatrilat significantly reduced the composite end point of death, admission or discontinuation of study treatment for worsening HF compared to the ACE inhibitor lisinopril (Rouleau et al. 2000). The more favorable results in the IMPRESS trial may be due to the fact that patients enrolled in this trial had on average less severe HF.

A potential problem of NEP inhibition is that it could also inhibit the degradation of endothelin, a potent vasoconstrictor and also a NEP substrate. More upstream from NEP in the processing of BNP is DPP-IV which was reported to cleave BNP 1–32 to BNP 3–32 in vitro (Brandt et al. 2006). In healthy canines, synthetic BNP 3–32 as compared to BNP 1–32 had no vasodilating actions and had reduced natriuretic actions (Boerrigter et al. 2007b). No reports are available regarding the effect of DPP-IV inhibition on endogenous and exogenous BNP levels in vivo, which may be important to know particularly because the first DPP-IV inhibitor, sitagliptin, has recently been approved for the treatment of type II diabetes mellitus. Meprin A cleaves BNP 1–32 to BNP 8–32, which then also becomes a substrate of NEP (Pankow et al. 2007). BNP 8–32 in healthy canines compared to BNP 1–32 had similar hemodynamic but reduced natriuretic actions (Boerrigter et al. 2008). Based upon these results one could speculate that enzymatic degradation of the NPs not necessarily only reduces their bioactivity, but it may also modify their profile of actions.

5 Phosphodiesterase Inhibition

The cGMP signal is effectively terminated by the action of specific phosphodiesterases that hydrolyze cGMP to GMP. Inhibition of the isoenzyme PDE5A (e.g. with sildenafil, vardenafil, tadalafil) reduces the vascular tone particularly in the corpus cavernosum and the pulmonary vasculature, leading to its application in

erectile dysfunction and pulmonary arterial hypertension. In a murine model of pressure overload due to aortic banding, chronic sildenafil (100 mg kg^{-1} body weight) attenuated cardiac hypertrophy, dilation, and fibrosis, and improved cardiac function (Takimoto et al. 2005). Indeed, cardiac hypertrophy was not only prevented but, once established, could be reversed with sildenafil. This was associated with decreased activation of hypertrophic factors such as calcineurin, the mitogen-activated kinase ERK1/2, Akt, and PI3Kα. However, a different group using the same animal model reported that sildenafil (154 ± 5 mg kg^{-1} body weight per day) did not prevent hypertrophy and actually decreased ejection fraction (Eder et al. 2007). The reason for this discrepancy is unclear but may include the sildenafil dose and the degree of aortic banding. In canine pacing-induced HF, chronic PDE5 inhibition with sildenafil reduced systemic vascular resistance and increased cardiac output but did not affect cardiac filling pressures or renal function (Chen et al. 2006a). Of note, this model is not associated with the development of cardiac hypertrophy. Sildenafil post-myocardial infarction in mice reduced infarct size and apoptosis, improved left ventricular function 7 and 28 days post-MI, reduced cardiac hypertrophy, and improved survival (Salloum et al. 2008). It would be interesting to know whether short-term administration for only a few days post-MI would be sufficient to improve remodeling or whether chronic therapy is required.

In a small human study ($n = 34$) in systolic HF patients with secondary pulmonary hypertension (mean pulmonary artery pressure >25 mmHg), those randomized to sildenafil (25–75 mg orally 3 times daily for 12 weeks) demonstrated reduced pulmonary vascular resistance at rest and during exercise compared to placebo (Lewis et al. 2007). Peak cardiac output and maximal oxygen uptake during exercise also increased; while six-minute walk distance and symptomatic status improved. No significant change in mean arterial pressure, systemic vascular resistance, pulmonary capillary wedge pressure, or heart rate was observed. Of note, the sample size was small and slightly more, than half the patients were in NYHA class II. In another study, male HF patients ($n = 46$; NYHA class II or III) were randomized to sildenafil (50 mg thrice daily) or placebo and reevaluated after 3 and 6 months (Guazzi et al. 2007). Sildenafil led to sustained reduction of pulmonary artery systolic pressure, improved flow-mediated dilation of the brachial artery, improved peak oxygen uptake and exercise ventilation, and improved symptomatic status. No serious adverse events with sildenafil were obvious in either trial. These studies suggest that at a minimum PDE5 inhibition can improve functional status in HF patients. It remains to be determined what its effect on mortality is and how patients with more advanced disease respond.

6 Multivalency Strategies

Taking the individual strategies to augment cGMP systems one step further, one could combine two or more strategies (Fig. 1). Little is known about simultaneous activation of sGC and GC-A. As both pathways differ in their tissue and cellular targets, augmenting both may be a beneficial strategy. In canine pacing-induced

HF, combination of BNP and the NO- and heme-independent sGC activator BAY 58–2667 resulted in vasodilation, increased cardiac output and renal blood flow, and natriuresis, a profile not achieved by either compound alone (Boerrigter et al. 2007). In the NAPA trial which showed beneficial effects of perioperative nesiritide infusion during cardiac surgery in patients with impaired LV function, more than half the patients in the nesiritide arm also received a nitrovasodilator (Mentzer et al. 2007). Chen et al. reported that in canine pacing-induced HF after chronic PDE5 inhibition (see Sect. 5) (Chen et al. 2006a) the response to BNP was augmented. Interestingly, chronic PDE5 inhibition led to significantly increased plasma cGMP levels despite lower BNP levels, which would be consistent with a "leaking" sGC-dependent cGMP pool.

Currently, medication with nitrates is considered a contraindication for PDE5-inhibition, due to the potential of excessive hypotension; however, it may be worthwhile to formally test the cardiorenal actions of this strategy with correspondingly lower sGC stimulator doses. This may especially be the case in the cardiorenal syndrome that is characterized in a model of overt HF to be resistant to ANP in association with reduction in GFR and renal blood flow together with increases in glomerular PDE activities (Supaporn et al. 1996).

7 Conclusion and Future Directions

Cyclic GMP is a second messenger crucially involved in important signaling pathways in cardiovascular disease, including heart failure. Potent drugs are now available to augment these signaling systems with conventional sGC stimulators, novel NO-independent sGC stimulators, GC-A and GC-B agonists, and PDE inhibitors. Given some of the already available promising preclinical and clinical data, it is likely that the cGMP systems will remain attractive areas of research and drug development. Indeed, in strategies to treat heart failure, the focus has been on antagonizing endogenous neurohumoral systems. Cyclic GMP research has opened a new direction in HF therapeutics by providing us with an exciting way to promote signaling pathways that possess robust properties of cardiorenal protection warranting further basic and clinical research.

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Erectile Dysfunction and Lower Urinary Tract

Peter Sandner, Dieter Neuser, and Erwin Bischoff

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Abstract During the last decades it turned out that the NO/cGMP signaling cascade is one of the most prominent regulators of a variety of physiological and pathophysiological processes in a broad range of mammalian tissues. Thus cGMP is a key second messenger and targeting this pathway by increasing intracellular cGMP levels is a very successful approach in pharmacology as shown for nitrates, PDE5 inhibitors and more recently for stimulators of the guanylate cyclase. Besides the beneficial effects of cGMP elevation in cardiac, vascular, pulmonary, renal or liver

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disorders the launch of PDE5 inhibitors for the treatment of erectile dysfunction 10 years ago, has directed a lot of attention to the NO/cGMP signaling in the lower urinary tract. Triggered by the use of PDE5 inhibitors in ED it turned out that cGMP is a common regulatory mechanism for lower urinary tract function also beyond ED. In recent years intense research and development efforts were undertaken to elucidate the role of the NO/cGMP and to fully exploit the therapeutic implications of cGMP elevation in urological disorders in ED and beyond. Therefore we have summarized the effects of cGMP elevation for treatment of erectile dysfunction in males and in females. We have also reviewed the recent pre-clinical and clinical lines of evidence for treatment options of benign prostatic hyperplasia and lower urinary tract symptoms in male patients and overactive bladder and urinary incontinence in female patients. In addition we also touch more speculative concepts using cGMP elevating drugs for the treatment of premature ejaculation, peyornies disease and stone disease.

Keywords: Lower urinary tract symptoms · Overactive bladder · Erectile dysfunction · Femals sexual dysfunction · PDE5 inhibitors · Vardenafil
Declaration of Interest:
All authors are employees of Bayer Schering Pharma

1 Introduction

The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), although discovered decades ago, still represent one of the most important second messenger pathway within cells. It is well established that the regulation of intracellular cAMP and cGMP pools have significant impact on physiology and pathophysiology and is one basic principle of pharmacological intervention.

After the discovery of nitric oxide (NO), it turned out that NO could trigger cGMP elevation via stimulation of the soluble guanylate cyclase (sGC). In addition, the natriuretic peptides could increase intracellular cGMP via activation of the membrane-bound, particulate guanylate cyclase. In the meantime, the signaling cascade starting at the formation of NO to the downstream targets of cGMP has been elucidated and the mode of action of cGMP is established. In brief, NO is formed from L-arginine via the activity of NO-synthases of which three iso-enzymes exist. Consecutively, NO activates the sGC resulting in enhanced cGMP formation. In the next step, cGMP regulates different downstream targets, mainly cGMP regulated protein kinases (G-kinases), cGMP-regulated phosphodiesterases (PDEs), and cGMP regulated ion channels. Finally, the rise in cGMP is translated into a decrease of intracellular free calcium levels. Thus, one of the most prominent physiological responses of increasing intracellular cGMP, especially in the smooth muscle cells (SMC), is relaxation of these cells. In addition, antiproliferative, antifibrotic,

or proapoptotic effects of cGMP are discussed. The above described signal transduction pathway, often referred as NO/cGMP pathway, is pharmacologically targeted at different levels. L-arginine, NO-donors, natriuretic peptides, PDE5 inhibitors, and very recently also sGC stimulators and sGC activators are used. These compounds increase intracellular cGMP via different mechanisms. Whereas arginine is used to increase the amount of NO production via the NO synthases, NO-donors directly mimic the action of NO. PDE inhibitors increase the intracellular cGMP levels by blocking the PDE-induced hydrolysis of cGMP via cGMP-phosphodiesterases. More recently, sGC stimulators and activators were used, which are able to directly stimulate the sGC triggering cGMP production.

The clinical introduction of the PDE5 inhibitors, which potently and selectively inhibit cGMP breakdown, was a landmark for the treatment of male erectile dysfunction (ED). Therefore, the launch of Viagra® (sildenafil) 10 years ago, illustrated for the first time the functional impact of cGMP increase in penile tissue. In the meantime, other target tissues in the lower urinary tract were identified in which the NO/cGMP cascade plays a functional role. It was shown that cGMP regulates ureteral tone, bladder, and prostate contractility but also relaxes female genital tissues. Therefore, intense research and development efforts are currently under way to treat diseases of the lower urinary tract by targeting the NO/cGMP system. Thus, we have summarized the pharmacological approaches targeting the NO/cGMP pathway for ED and for other diseases of the lower urinary tract and will discuss treatment options based on cGMP.

2 Male Erectile Dysfunction (ED)

Since almost 50% of men over 50 years are reporting some degree of erectile dysfunction (Melmann and Gingel 1999), ED represents a highly prevalent disorder in the aging male population. The prevalence rates of clinically relevant ED are about 20% worldwide with 22% and 19% in the United States and Europe, respectively (Braun et al. 2000; Laumann et al. 2007). ED is a neurovascular phenomenon modulated by hormonal, local biochemical, and structural factors of the penis. Today, ED is understood to represent predominantly organic aetiologies. It has a clear association with diabetes and cardiovascular diseases like hypertension or coronary heart disease (CHD) and may serve as a harbinger of later cardiovascular events (Thompson et al. 2005). It is estimated that the number of patients will increase from 150 millions in 1995 to more than 300 millions in 2025 (Ayta et al. 1999). The current official ED-market is already estimated to be *E*1.7 billion sales in 2004, mostly driven by the three marketed PDE5 inhibitors: Viagra® (sildenafil) approved in 1999; Levitra® (vardenafil) and Cialis® (tadalafil) both approved in 2003.

2.1 PDE5 Inhibitors

2.1.1 Preclinical Rationale and Mode of Action

During last decade, the basic understanding of ED has become very clear and is covered by variety of scientific publications and also broadly reviewed (De Tejada et al. 2004; Carson and Lue 2005; Francis and Corbin 2005). Various neurotransmitters including NO, oxytocin, and dopamine are recognized as pro-erectile neurotransmitters, necessary for penile erection. Nitric oxide, either during direct or psychogenic sexual stimulation, is synthesized by neuronal NO synthase (nNOS) in the nerve terminals of parasympathetic, non-adrenergic, non-cholinergic (NANC) neurons in the penis and also by endothelial NO synthase (eNOS) in the endothelial cells of the blood vessels and the lacunar spaces of the corpora cavernosa activates smooth muscle cell soluble guanylate cyclase (sGC) (Francis and Corbin 2005). This NO-production results in increased intracellular cGMP levels, which leads to relaxation of smooth muscle in the corpus cavernosum and in penile arterioles. Relaxation of arterial smooth muscle is accompanied by increased blood flow to the penile corpora. Trabecular smooth muscle relaxation leads to the opening of the sinusoids in the penile erectile tissue, which is the prerequisite for the initiation of an erection. The level of cGMP is regulated by its rate of synthesis via guanylate cyclase (sGC) and its hydrolysis to the physiologically inactive GMP by the cGMP-hydrolyzing phosphodiesterases. Of all cGMP hydrolyzing PDEs, PDE5 is the most prominent in the human corpus cavernosum, but some PDE2 activity (Boolell et al. 1996) and as significant amounts of the cAMP-metabolizing PDE3 and PDE4 (Taher et al. 1992, 1997) has also been described. Inhibition of PDE5 leads to an increase in the level of cGMP, enhancing relaxation of smooth muscle. Consequently, the vascular tone in penile arteries decreases. This causes increased blood flow and an enlargement of the cavernosal tissue, which induces penile erection. (Schultheiss and Stief 1999; Lue 2000). NO-mediated vasorelaxation is the basis for the therapeutic application of PDE5 inhibitors in the treatment of ED. This signaling pathway and the enhancing effect of NO on erectile function was demonstrated in isolated corpus cavernosum (Rajfer et al. 1992) and various animal models of different species as well as the effects of amplification of NO by administration of PDE5 inhibitors (Trigo-Rocha et al. 1993; Bischoff and Schneider 2001; Giuliano et al. 2003; Holmquist et al. 1991). The release of NO by NANC nerves and/or of the endothelium is impaired under pathological conditions, such as diabetes and/or coronary heart disease, hypertension, or spinal cord injury, which consequently leads to reduced cGMP synthesis. However, through PDE5 inhibition, sufficient levels of cGMP leading to erection in many patients with ED can still be reached – which is clinically important in patients with, e.g. diabetes, where impaired NO release might be the cause of the ED. A prerequisite to PDE5 inhibition as a therapeutic principle for the treatment of ED is sexual stimulation leading to the release of NO as described above. However, PDE5 is substantially expressed in many other tissues throughout the body. The selective induction of vasorelaxation in penile tissue is predominantly based on the increased synthesis of cGMP

in the cavernosal tissue in the presence of NO, which occurs only during sexual stimulation. This unique situation of increased cGMP concentrations after neural stimulation contributes more strongly to the selective cavernosal vasodilation (Murray 1993; Francis and Corbin 2005). This fact provides the basis for the efficacy and favorable therapeutic benefit versus side effect ratio, which is found for PDE5 inhibitors in the treatment of ED.

2.1.2 Clinical Results

Currently, there are three PDE5 agents commercially available worldwide for the treatment of ED: sildenafil (Viagra®), tadalafil (Cialis®), and vardenafil (Levitra®). Two new PDE5 inhibitors udenafil, (Zydena®) and mirodenafil (M-vix®) entered the Korean market in 2005 and 2007, respectively. With respect to their in vitro potency, vardenafil is the most potent compound, 25 times more compared to sildenafil and 45 times to tadalafil (Gbekor et al. 2002). All three compounds are highly selective for PDE5. Sildenafil and vardenafil show some cross reactivity for PDE6, and tadalafil shows some cross reactivity for PDE11. The most salient difference among the three compounds is the very long half-life time of tadalafil (17.5 h), compared with sildenafil (3–4 h) and vardenafil (4–5 h). Clinically, this difference is reflected in duration of action for sildenafil and vardenafil between 8 and 12 h compared to 36 h for tadalafil (Mehrotra et al. 2007).

All three drugs are highly effective in enhancing erectile function across a wide range of outcome measures, causes of ED patient subgroups and regional populations. In comparing clinical efficacy of the PDE5 inhibitors, it is important to note that most published studies for each compound reveal a comparative evaluation with placebo and not to each other. Only few studies exist, where a direct comparison of individual PDE5 inhibitors against each other is made. All three PDE5 inhibitors are distinctly superior compared to placebo, when the standard efficacy endpoints are used. The most relevant point in efficacy from the patient perspective is success in completing intercourse. The outcome was similar for all drugs across many studies, ranging from 60 to 70% for the recommended starting dose for each PDE5 inhibitor. (Sadovsky et al. 2001; Chen et al. 2001; Seftel 2004; Porst et al. 2003; Hellstrom et al. 2002) Differences in efficacy between individual PDE5 inhibitors are small and are more driven by the different pharmacokinetic profiles than by pharmacological potency. Ultimately, the patient makes the choice and selects the product, where he has the best experience with (Hatzichristou et al. 2007; Dunn et al. 2007). In patients with underlying conditions, in which NO release is impaired or destructed, e.g. in spinal cord injured or severe diabetic patients, PDE5 inhibitors may show insufficient efficacy.-(De Tejada 2004).

2.1.3 Adverse Events and Safety

The high efficacy of PDE5 inhibitors in ED is accompanied by high tolerability and safety of this class of drugs (Hellstrom 2007). Adverse events caused by PDE5 inhibitors are related to relaxation of SMCs in other parts of the body that express PDE5. Headache, flushing, dyspepsia, and nasal congestion have been reported in varying degrees for all three drugs (Seftel 2004; Rashid 2005). Visual disturbances, sometimes seen at the highest dose of sildenafil may reflect a partial inhibition of PDE6. Discontinuation rates due to AE's(no expanded form given) are low (2.1–2.5%) and similar for all three drugs. All PDE5 inhibitors may potentiate the hypotensive effects of organic nitrates (Rashid 2005) and therefore, they are contraindicated for patients using nitrate therapy.

Cardiac safety has been discussed in relation to PDE5 inhibitors and has been a subject of great interest. Clinical trials and post marketing surveillance have demonstrated no increase in myocardial infarction in patients, who received these agents compared to expected rates in age matched populations. Furthermore, numerous studies have proven not only the cardiovascular safety of PDE5 inhibitors but have also described mechanisms and clinical results of possible cardiovascular benefits of PDE5 inhibitors (Kostis et al. 2005; Carson 2005; Kukreja et al. 2007).

After almost a decade of use in clinical practice, PDE5 inhibitors can be considered as a very safe class of drugs, when used according to the labeling instructions (Hellstrom 2007).

Nonarteric anterior ischemic optic neuropathy (NAION) has also become a concern because post marketing surveillance has detected >50 cases of NAION among PDE5 inhibitor users. Another concern relates to the possibility of tachyphylaxia, which describes the rapidly decreasing response to a drug therapy. However, tachyphylaxia is unlikely since decreased efficacy of PDE5 inhibitors reflects the progression of underlying diseases rather then tachyphylaxia (Gonzalgo et al. 2003).

2.2 Other NO/cGMP Approaches for ED

Although the class of PDE5 inhibitors represent a highly efficient treatment option for ED, 20–30% of ED patients do not respond to PDE5 inhibitors. This is at least in part due to a limited endothelial or endothelial cell derived NO-production by eNOS and nNOS caused, i.e. by severe diabetes or radical prostatectomy. Clinical effectiveness of PDE5 inhibition needs at last a minimal NO signal in order to induce some turnover of cGMP. Otherwise, PDE5 inhibition will not be able to increase intracellular cGMP and induce vasorelaxation. Complete destruction of the cavernosal nerves during radical prostatectomy or severe diabetic neuropathies could be pathological conditions, which principally limit the success of treatment with PDE5 inhibitors (De Tejada 2004). Therefore, new treatment principles within the NO/cGMP system, which actively stimulate cGMP production in the corpus cavernosum (e.g. with NO-donors) are another avenue of research and might be also able to overcome PDE5-resistent ED.

2.2.1 L-Arginine and NO-Synthase Modulators in ED

A very first step in the NO/cGMP cascade is the formation of nitric oxide via conversion of L-arginine to citrullin by NO-synthase. However, an attempt to use L-arginine for the treatment of ED failed in a clinical study (Chen et al. 1999). More successful and also tested clinically was a compound, which is an NOS co-factor that showed efficacy in human ED patients (Esteve Laboratorios 2004). In addition, a gene therapy approach, injecting plasmids either expressing iNOS or nNOS in the rat penis was tested (Garban et al. 1997; Magee et al. 2002). A preclinical study in diabetic streptozotocin (STZ) treated rats demonstrated that the erectile response of eNOS gene therapy along with sildenafil was greater than in the STZ-rats receiving sildenafil or eNOS gene therapy only (Bivalacqua et al. 2004). Apart from this encouraging preclinical result, a careful analysis of gene therapy approach for the treatment of ED is necessary (Kendirici et al. 2005).

2.2.2 NO-Donors

Reports about the impact of NO-donors for the treatment of ED are scare. Stief et al. showed already in 1992 that injection of the NO-donor, SIN-1, in ED-patients intracavernously, resulted in erections (Stief et al. 1992). It has been shown that SNP and the new NO donor, S-Nitroso-glutathione (GSNO) could relax human corpus cavernosal tissue to the same extent than sildenafil (Seidler et al. 2002). In contrast, injection of these compounds in human corpus cavernosum did not change penile blood flow significantly (Fu et al. 2001). In summary, the treatment of ED with NO-donors had not reached clinical relevance for a broader population.

2.2.3 sGC Stimulators and Activators

A very interesting class of drugs – not only for ED treatment – could be NO-independent stimulators of the soluble guanylate cyclase (Evgenov et al. 2006), such as YC-1 (Ko et al. 1994), BAY 41–2272 (Stasch et al. 2001), or A-350619 (Miller et al. 2003). In addition to the action on cardiovascular functions, there is recent evidence that these compounds could be used for ED treatment. One of the first compounds activating the sGC was YC-1 (Ko et al. 1994), which also inhibited the PDE5 (Friebe et al. 1998). The effects of YC-1 on erectile function have been tested in rats, where direct intracavernosal injection enhanced erections (Mizusawa et al. 2002). Additionally, A-350619, another sGC stimulator has been shown to induce penile erections in a rat model of erectile dysfunction (Miller et al. 2003). Recently, more advanced compounds were developed activating the sGC, especially BAY 41–2272, which was more potent compared to YC-1 and also did not show PDE5 activity (Stasch et al. 2001; Straub et al. 2001; Bischoff and Stasch 2004). BAY 41–2272 could relax human and rabbit cavernosal tissues in vitro (Kalsi et al. 2003; Baracat et al. 2003). The same compound also induced stable erections in

rabbits after intravenous and oral applications in vivo (Bischoff et al. 2003). In addition, it was demonstrated that activation of the sGC with BAY 41–2272 could have advantages in rats with severe diabetes-induced ED in which PDE5 inhibitors were of moderate efficacy (Kalsi et al. 2004). These results show, that increase of cGMP via stimulation of the sGC could be an alternative treatment option to PDE5 and might be beneficial in difficult-to-treat patients. It is an intriguing hypothesis that sGC stimulators might be superior compared to PDE5 inhibitors in ED since they are not solely dependent on endogenous NO-induced cGMP production, which is impaired in severe diabetes or after radical prostatectomy. However, there is more preclinical, and clinical data needed, if these compounds will have higher efficacy in difficult-to-treat ED patients. In addition, based on the ubiquitous distribution of sGC in different tissues, the effects of such type of compounds on systemic blood pressure have to be carefully monitored in terms of their risk/benefit profile for ED treatment.

2.2.4 Combination Therapy

One possible alternative for ED treatment, especially for those patients not responding to PDE5, might be to combine several NO/cGMP mechanisms, i.e. an NO-donor and a PDE5 inhibitor. Schwarz Pharma claimed a combination of the NO-donor, pentaerythrityltetranitrate (PETN) and PDE5 inhibitors for the treatment of impotence and coronary heart disease (Schwarz Pharma 2000). However, one has to keep in mind that mechanisms which synergistically elevate cGMP levels could raise a safety concern since it could cause hypotension. In fact, preclinical data shows that the combination of PETN and sildenafil induced a significant drop in the blood pressure in dogs. This side effect might become a general limitation for combining PDE5 inhibitors with other enhancers of the NO-cGMP axis but also with other agents inducing relaxation of smooth muscle cells. This will also be a limitation of compounds acting as PDE5 inhibitor but also releasing NO, like NCX-911 (Kalsi et al. 2005). It was reported in recent clinical trials that responding to PDE5 inhibitors is dependent on the serum testosterone levels. PDE5 inhibitors are less effective in a hypogonadal status compared to the effects in patients receiving testosterone substitution (Shabsigh et al. 2004, 2005). The reason for this observation is not fully understood, however, this might be due to interference of testosterone with the NO/cGMP pathway, which is currently under investigation (Martina et al. 2006; Waldkirch et al. 2008).

2.3 Summary and Conclusion

Taken together, the impact of cGMP increase for treatment of ED is well established and PDE5 inhibitors are the gold standard for the treatment to date. There might be some additional developments, within the field of NO/cGMP pathways, in the future

ED treatment. Especially, for the difficult to treat patients using NO-independent stimulators of cGMP production could be one very successful approach in the future.

3 Female Sexual Dysfunction (FSD)

In contrast to the broad knowledge of male penile function, the physiology and pathophysiology of female sexual dysfunctions (FSD), also often referred as female sexual arousal disorders (FSAD) is not very well understood. Moreover, the role of the NO/cGMP for FSD and FSAD needs to be clarified. It is hypothesized that cGMP increase could be useful in the treatment of FSD and FSAD via stimulation of genital blood flow, relaxation of the smooth muscle of the vagina and clitoris, finally facilitating penile penetration and sexual stimulation during sexual intercourse (Bermann and Bassuk 2002; Khan et al. 2000). Therefore, the expression and the functional role of the NO/cGMP systems in the female genitals was studied intensively in recent years. It has been shown that the eNOS and nNOS are abundantly expressed in the human clitoris (Burnett et al. 1997b) and that inhibition of NO production with L-NAME decreased female vaginal blood flow in the rat (Kim et al. 2004). Since PDE5 inhibitors are a very safe and effective method to increase cGMP, the use of PDE5 inhibitors was investigated. The expression of various cGMP degrading PDEs, including PDE5, have been demonstrated in human female genital tissue by immunohistochemistry and mRNA expression profiling (Uckert et al. 2006, 2007). Moreover, on the functional level, the occurrence and hydrolytic activity of the PDE-5 was shown in human clitoral corpus cavernosum and human vagina (Park et al. 1998; Uckert et al. 2005a, b; Oelke et al. 2006). Finally, organ bath experiments demonstrated that PDE-5 inhibitors are able to relax human vaginal wall muscles (Uckert et al. 2005a, b) and also clitoral muscles of rat (Tinel et al. 2006a). The effects of PDE5 inhibitors on rat vagina and clitoris were even more pronounced than on male corpus cavernousom (Tinel et al. 2006a). Therefore, PDE-5 inhibitors are thought to improve vaginal and clitoral blood flow and facilitate arousal and orgasm in women. In addition, it was shown that vardenafil enhances prospective and receptive behavior in female rats (Tinel and Sandner 2008). However, the results of clinical trials using sildenafil were not very encouraging. Sildenafil showed only moderate effects to most women with FSD (Caruso et al. 2001; Basson et al. 2002; Basson and Brotto 2003; Berman et al. 2003). The studies showed that PDE-5 inhibition might be of benefit for individuals with female sexual arousal disorder (FSAD) but not with hypoactive sexual desire disorder (HSDD). Since FSAD is mostly common in women suffering from diabetes, this patient group might respond more effectively to PDE5 inhibitors (Enzlin et al. 2002). In fact, it has recently been shown that sildenafil improved sexual function in premenopausal women with diabetes (Caruso et al. 2006). Thus, it seems that some patient groups might profit from a treatment with PDE-5 inhibitors, but new studies are necessary to investigate it in detail. In addition, the linear model of

sexual response which is true for men, disregards the relative independence between subjective and objective aspects of women's sexual response (Laan et al. 1995; Althof et al. 2005).

While planning new studies, one should take into consideration also these new findings concerning female sexuality. Taken together, increasing cGPM via PDE-5 inhibitor treatment might be of benefit to special patient groups, but new clinical studies with a modified design are necessary to reveal the impact of the NO/cGMP system and also the use of PDE5 inhibitors (Claret et al. 2006).

4 Benign Prostate Syndrome (BPH) and Lower Urinary Tract Symptoms (LUTS)

"BPH" benign prostatic hyperplasia, more appropriately termed benign prostatic syndrome (BPS), since hyperplasia is not diagnosed in every case, is a highly prevalent disorder in the aging male. BPS comprises benign prostatic enlargement (BPE) with different degrees of bladder outlet obstruction (BOO) and lower urinary tract symptoms (LUTS) (Roehrborn 2002). BPS is affecting 50% of the male population over 50 years and considerably decreases the quality of life of the patients (Guess 1995; Thorpe and Neal 2003). Moreover, it is also a high economic burden for the health care systems, since one out of four men is looking for medical treatment for relief from BPS (Jacobson et al. 1993). The lower urinary tract symptoms (LUTS), although not life-threatening, are very bothersome for the patients and could be divided into obstructive and irritative symptoms. Obstructive symptoms, often referred as voiding symptoms, are arising from the compression of the urethra by the enlargement of the prostate (BPE) and include slow stream, intermittency, dysuria, and dribbling. The irritative symptoms, often referred as storage symptoms, comprise frequency, urgency, incontinence, and nocturia (Abrams 1994). The current pharmacotherapy is dominated by the use of alpha-blockers like tamsulosin, alfuzosin, and doxazosin, which decrease the symptom-rate by dilatation of prostate tissues via blockade of the alpha-1 receptor. Moreover, 5-alpha-reductase inhibitors like finasteride and dutasteride are used (Carbone and Hodges 2003; McVary 2007). Since pharmacotherapy is of limited efficacy and also exhibit side effects like dizziness, retrograde ejaculation for alpha blockers, and erectile dysfunction for 5-alpha-reductase treatment, there are intense efforts of preclinical and clinical research to improve pharmacotherapy by either introduction of new treatment principles. Since it is known that cGMP could relax smooth muscles, this could be also relevant for dilatation of the prostate and the bladder. Moreover, there are some results implying that cGMP might also attenuate cellular proliferation. Therefore, one very exciting recent treatment approach for LUTS and for BPE is to increase cGMP levels within the prostate and the bladder.

4.1 Expression and Functional Relevance of the NO/cGMP/PDE System in the Prostate

The description of the NO/cGMP/PDE system, including NO-synthases, G-kinases, and PDE5 in bladder and prostate tissues lagged behind, if compared to other organ systems. Nevertheless, there was a very first report detecting PDE activity in the human prostate in 1970 (Kuciel and Ostrowski 1970), followed by indirect evidence that enhancement of cGMP formation via NO-donors resulted in relaxation of human, dog, and rabbit prostate strips (Takeda et al. 1995; Hedlund et al. 1997). Prostatic tissue from BPH-patients express all three iso-forms of NO-synthases and display nitric oxide synthase activity (Hedlund et al. 1997; Najbar-Kaszkiel et al. 1997; Gradini et al. 1999) and more recently, it was shown that also G-kinase 1-alpha and 1-beta are localized in the transition zone of human prostates (Waldkirch et al. 2007). In 1998, when human PDE5 was cloned and described (Loughney et al. 1998; Yanaka et al. 1998; Stacey et al. 1998), it became clear that there was also PDE5 expression in the prostate and in the bladder (Stacey et al. 1998). In 2001, Uckert et al. identified mRNAs of different PDE iso-genes including the cGMP-specific PDE5 mRNA in the human prostate (Uckert et al. 2001). It was also demonstrated by the same group that cGMP elevation with either NO-donation or PDE5 inhibition via zaprinast and sildenafil could relax human prostatic tissue (Uckert et al. 2001). The relaxation of prostatic tissues by sildenafil was also observed in normal and ischemic prostates of rabbits (Azadzoi et al. 2003). More recently, a direct comparison of sildenafil, vardenafil, and tadalafil was done in the rat prostate showing a dose-dependent relaxing of these PDE5 inhibitors revealing a rank order of potency of vardenafil > sildenafil > tadalafil (Tinel et al. 2006b). All these data suggest that PDE5 is expressed in prostates of humans and rodents, and PDE5 inhibition could directly dilate prostatic smooth muscle via cGMP, which could influence the dynamic component of obstruction. In a very recent work, the localization of PDEs was investigated within the human prostate more precisely. There was a significant immunostaining of PDE5 observed in the fibromuscular stroma, and – even in a higher density – within the whole peri-urethral glandular region, including glandular and sub-glandular regions of the transition zone (Uckert et al. 2006). The human prostate strips, which were relaxed by zaprinast and sildenafil were also derived from the transition zone (Uckert et al. 2001). This expression profile implies a functional role for PDE5 in regulation of stromal smooth muscle tone as glandular secretory function and proliferation. Moreover, these are also the regions where alpha-1 receptors are expressed mediating the effect of alpha blockers on the prostate (Hieble and Ruffolo 1996), strongly supporting the concept that PDE5 inhibitors could act in the same direction than alpha-blockers. In fact, this was shown recently in integrative in vivo studies. As an animal model, mimicking prostate hyperplasia, rabbits, rat, and mice with partial bladder outlet obstruction (BOO) were used. After partial ligation of the urethra with a silk, within 2–6 weeks these animals develop a bladder hypertrophy, a decreased bladder contractility, and non-voiding contractions of the bladder, which could be detected by cystometry. The

non-voiding contractions are taken as correlation of irritative symptoms reflecting lower urinary tract symptoms in patients. In the rabbit, partial bladder outlet obstruction increased bladder weight and decreased bladder contractility; however, blocking the NO-synthesis with L-NAME hat bladder protective effects (Conners et al. 2006). This result has to be interpreted carefully, since it does not necessarily imply that cGMP might have negative impact on bladder function and irritative symptoms in this BOO model, rather then NO-derived radicals exert this negative effect. In fact, it has shown that cGMP increase with vardenafil and sildenafil reduced non-voiding contractions after acute bolus application in BOO rats (Tinel et al. 2006b). In addition, it was also found that a chronic 4-week treatment with vardenafil, significantly reduced the numbers of non voiding contractions in BOO rats indicating a reduction of irritative symptoms (Filippi et al. 2007). These results were extended by findings of Matsumoto and coworkers, showing that a 4-week vardenafil treatment of BOO rats restored the contractile response in the bladder, implying that vardenafil had bladder protective effects (Matsumoto et al. 2007). Finally this was confirmed by findings in mice, where a 6-week oral sildenafil treatment also showed bladder protective effects. Interestingly in this study, sildenafil also decreased bladder muscular hypertrophy and bladder collagen levels and resulted in an increase of the bladder capacity compared to placebo (Beaman et al. 2007), which was not reported from the other studies. Taken together, these results in BOO rats and mice strongly suggest that vardenafil and sildenafil by their ability to increase prostatic cGMP levels, reduce bladder-derived symptoms in obstructed animals.

4.2 The Role of NO/cGMP/PDE for Prostatic Proliferation and Hyperplasia

There could be an additional avenue for cGMP making them a treatment option for BPE and BPH, which could cause LUTS. Up to now, we have considered the dynamic component of the disease, which could be influenced by prostate relaxation. However, there is also some evidence that cGMP and PDEs could influence proliferation of tissues, which might have impact on the static component of obstruction. It has been shown that PDE5 inhibitors could reduce cardiac hypertrophy (Takimoto et al. 2005) or proliferation of pulmonary artery smooth muscle and endothelial cells (Wharton et al. 2005). For prostatic tissues, data on proliferation are relatively scarce. For benign prostatic tissues, there was an early report in 1998, that cGMP accumulation induced by NO-donor SNP and the cGMP analogue 8-Br-cGMP was able to inhibit the proliferation of human prostate smooth muscle cells (Guh et al. 1998). In 2002 it was published, that either NO-donors (SIN-1, SNAP) and also the PDE5 inhibitor sildenafil could inhibit proliferation of cultured smooth muscle derived from human prostate tissues (Adolfsson et al. 2002). This was confirmed later on in cultured human prostatic stromal cells, using the cGMP analogue, 8-pCPT-cGMP and the PDE5 inhibitor, zaprinast (Cook and Haynes 2004, 2007). Both compounds showed a dose-dependent decrease of proliferation, which could

be blocked by the G-kinase inhibitor, Rp-8-BrcGMP (Cook and Haynes 2004). More recently, it has been shown that all three marketed PDE5 inhbitors, attenuate human prostate stromal cell proliferation with a rank order of potency of vardenafil > tadalafil > sidlenafil (Tinel et al. 2006b). In all these above mentioned experiments, different cell types (i.e. smooth muscle cells, stromal cells), different stimuli (i.e. fetal calf serum, lysophospathidic acid), and different time frames (24–96 h) were used. This makes it very difficult to draw conclusions, if these in vitro findings will translate to prostate mass reduction in vivo. Up to now – to our knowledge – there are no animal experiments published, which are looking on prostate growth and proliferation after chronic NO-donation or PDE5 inhibitor treatment. Nevertheless, the obtainable cellular data imply an anti-proliferative effect, which would be in fact a very intriguing feature of cGMP elevation, combining the anti-proliferation and relaxation in one mechanism, substantially influencing the dynamic and static component of obstruction in BPH and BPE.

4.3 Clinical Results with PDE5 Inhibitors in Symptomatic BPH

Since PDE5 inhibitors very effectively increase intracellular cGMP, and are also a quite safe class of drugs – widely used for the treatment of erectile dysfunction – these compounds were recently used in Phase II studies in patients with symptomatic BPH.

Sildenanfil, tadalafil, and vardenafil significantly reduced LUTS, which are described by the international prostatic symptom score (IPPS) (Kaplan and Gonzalez 2007). The IPSS, which includes obstructive and irritative scoring, was significantly reduced when BPS patients were treated once daily with 100 mg Viagra® (Mc Vary and Monning et al. 2007) or 20 mg Cialis® for 12 weeks (McVary et al. 2007b). In addition, a randomized, placebo-controlled study was conducted with Levitra in BPH patients. With 10 mg Levitra® BID, a significant improvement of the IPSS-score was detected (Stief et al. 2008). These studies demonstrated that PDE5 inhibitors could be an additional treatment option for patients suffering from LUTS.

5 Overactive Bladder (OAB) and Urge Urinary Incontinence (UUI)

Overactive bladder (OAB) is a term which was defined by the International Continence Society and describes bothersome bladder syndrome, comprising urgency with or without incontinence, frequency, and nocturia (Abrams et al. 2002). Urgency refers to the sudden and compelling desire to pass urine and could also be associated by incontinence episodes, termed urge urinary incontinence (UUI). In addition, patients' micturition frequency in these patients is creased with more than 8 voidings

during day-time and with more than 1 voiding during night time. UUI is the most bothersome symptom of OAB and is associated with a significantly reduced quality of life of the patients (Milsom et al. 2001). The etiology and pathophysiology of this disease is not completely understood and needs to be further elucidated. The voiding process is coordinated by neural circuits, including the spinal cord and the brain, which regulate the activity of the smooth muscle in bladder and urethra. Moreover, direct contraction and relaxation of the bladder smooth muscle plays a critical role in this disease (Andersson and Arner 2004) and also ischemia of the bladder muscle could contribute to OAB behavior. The main treatment option is based on muscarinergic-receptor antagonists (antimuscarinics), which prevent acetylcholine-induced contraction of the detrusor muscle during filling phase by blocking postsynaptic M-3 receptors (Chapple et al. 2005; Sahai et al. 2006b).

5.1 Expression and Functional Relevance of the NO/cGMP/PDE System in the Bladder

There is some evidence that the NO/cGMP system including phosphodiesterases (PDEs) in general, but also especially PDE1 and PDE5 inhibitors, could be used for the treatment of OAB. An early report demonstrated that the micturition frequency in rats was increased by blocking NO-production with L-NAME (Persson et al. 1992). In addition, transgenic nNOS KO-mice have higher number of micturitions compared to wild type mice (Burnett et al. 1997a). Moreover, it has been shown that different PDE isogenes and also PDE5 are expressed in the detrusor muscle (Truss et al. 1996a). Recently, PDE5 activity has been detected in the human bladder tissue and PDE5 was localized in the smooth muscle of the bladder (Filippi 2007). On the functional level, NO-donors and PDE5 inhibitors reduced the tonus of the bladder muscle in vitro of rat, rabbit, and human tissues (Truss et al. 1996b; Qui et al. 2001, 2002; Uckert et al. 2002). In addition, the PDE5 inhibitors vardenafil and sildenafil could relax human bladder neck tissue (Filippi et al. 2007) and also human detrusor muscle (Behr-Roussel et al. 2008), extending and confirming previous reports. However, the exact functional role of the NO/cGMP/PDE5 system in bladder is still under debate since there might be effects on the urothelium and on bladder innervation too. In fact, Gillespie and coworkers localized considerable amounts of eNOS and of NO-inducible cGMP in urothelial, suburothelial, and intramural neurons of guinea pig bladders. Since these regions are important for bladder sensations and the micturition threshold cGMP might act on local neuronal pathways as well (Gillespie and Drake 2004; Gillespie et al. 2006).

5.2 Clinical Results

Clinical data about the impact of NO/cGMP system for the treatment of OAB is still missing. There is preliminary evidence about the PDE1 inhibitor, vinpocetine

in OAB patients (Truss et al. 2000). In this pilot study, 19 OAB patients who were not responding to standard therapy were treated with vinpocetine and 11 patients showed improved clinical symptoms or urodynamic parameters. Since OAB comprise urgency, frequency, and nocturia, LUTS in BPH patients is similar to UUI in OAB patients. Both LUTS and UUI symptoms are originated within the bladder and characterized by detrusor overactivity (Chapple and Roehrborn 2006). Thus, one might assume, if PDE-5 inhibition with sildenafil, tadalafil, and vardenafil, which reduced LUTS in BPH patients (Mc Vary et al. 2007a, b; Stief et al. 2008) might be able to reduce UUI symptoms in OAB patients. In fact, a pilot study using vardenafil 20 mg in spinal cord injured patients with OAB showed an increase in bladder capacity and increase of the reflex volume (Gacci et al. 2007). Thus, increase of cGMP i.e. via PDE inhibition could be one very promising approach for future therapy of OAB and UUI.

6 Premature Ejaculation (PE)

Premature or rapid ejaculation is a male sexual disorder with high prevalence rates of 5–30% (Aschka et al. 2001; Montague et al. 2004; Althof 2006). To date, the pathophysiology and especially, the underlying molecular mechanisms are only poorly understood. Currently, tricyclic antidepressants, serotonin reuptake inhibitors, or topical application of local anaesthetics are used (Hellstrom 2006), but there is no approved pharmacotherapy. Behavioural therapy and psychological counseling is also considered to be effective, either alone or in combination with drug treatment (Piedifierro et al. 2004). All these current treatment options are limited by their efficacy and to some extent, also by their side effects. Since the quality of life of PE-patients, especially of the partner of the patient is quite low, a lot of efforts are currently made to improve the knowledge about the pathophysiology of PE, to improve the therapy. It has been agreed that relaxation of smooth muscles within the vas deferens (VD) and seminal vesicles (SV) could attenuate ejaculation and could be a useful for the treatment of PE. It was shown that NO/cGMP could influence VD and SV contractility. NO-donors and different PDE inhibitors are able to relax SV and VD (Schultz et al. 1977; Bialy et al. 1996; Heuer et al. 2002; Machtens et al. 2002; Mancina et al. 2005). Moreover, it was reported that homozygous eNOS knock out mice have higher rates of ejaculations requiring less stimulation, when compared to WT-mice (Kriegsfeld et al. 1999). This demonstrates that the NO/cGMP/PDE system is involved in PE. In several clinical studies, the PDE5 inhibitors, sildenafil, tadalafil, and vardenafil were used to treat PE patients (McMahon et al. 2006; Wang et al. 2006). Within these studies, an increased intravaginal ejaculatory latency time (IELT) was found during PDE5 inhibitor treatment. Despite these promising clinical results, only one clinical trial – conducted with sildenafil – fulfilled the evidence based medicine criteria. In this trial, no significant increase in IELT was observed (McMahon et al. 2005, 2006). Thus, it might be important to extend the preclinical

research in regard to the impact of the NO/cGMP/PDE system, but also conduct clinical study with modified protocols, to elucidate the potential of PDE5 inhibitors for the treatment of PE.

7 Peyronies Disease (PD)

Peyronie's Disease (PD) is a disorder characterized by fibrotic plaques of the tunica albuginea of the penis. The prevalence rates are depending on definitions, and are estimated between 0.4 and 9% (Pryor et al. 2004; Mulhall et al. 2004). The cause of the fibrotic plaque in PD is unknown but it is hypothesized that trauma with excessive wound healing might be able to cause peyronies. In addition, infections and autoimmune diseases could also contribute to progression of this local fibrosis in the penis. However, the molecular mechanisms are poorly understood. Therefore, surgical treatment options are predominant, whereas pharmacotherapy is still in an experimental stage (Pryor et al. 2004). Since fibroblast proliferation and accumulation in the penis promote PD, the attenuation of fibroblast proliferation could be a successful treatment option. It has been demonstrated that the NOS/NO/cGMP system could be significantly involved in progression of the plaque development. Inhibition of iNOS activity resulted in excaberated fibrosis (Ferrini et al. 2002) and the NO donor SNAP could influence differentiation of fibroblasts in myofibroblasts (Vernet et al. 2002). In primary cultures of human PD-derived fibroblasts, the unspecific PDE inhibitor, theophyllin and also the PDE5 specific inhibitior, sildenafil reduced collagen I synthesis and myofibroblast differentiation and increased apoptosis (Valente et al. 2003). Very recently, it was demonstrated in a rat model of PD that vardenafil significantly decreased collagen I and III deposition and reduced the numbers of myofibroblasts in the PD plaque (Ferrini et al. 2006a, b). These data clearly demonstrated that cGMP increase via PDE5 inhibition could attenuate TGF-induced collagen production and fibrosis. Clinical data about the impact of PDE5 inhibitors for the treatment of PD are currently not available. This might be due to the fact, that current labeling excludes the use of PDE5 inhibitors for PD patients. Nevertheless, the above mentioned data indicate that cGMP elevation, i.e. with PDE5 inhibitors, could attenuate penile fibrosis, rather than reverse it. In regard to the high medical need, it should be therefore tried carefully to confirm the in vitro and in vivo preclinical hints within clinical trials.

8 Stone Disease

Expulsion of urinary stones by facilitating uretheral passage of the stones would be an attractive opportunity for the use of cGMP increasing agents. So far, expulsion of the stones often accompanied by colic pain is treated with analgesics and combined with muscle relaxants e.g. alpha blockers (Singh et al. 2007). But there

is also evidence that the NO/cGMP axis plays a role in uretheral contractility. Neurons and nerve endings, expressing NO synthase have been detected in muscular layers of the human ureter and it was hypothesized that ureteral relaxation is also triggered by the NO/cGMP system (Stief et al. 1996). In fact, Iselin and coworkers could show that nitric oxide inhibited contraction of isolated pig ureteral muscle and also isolated human ureter (Iselin et al. 1996, 1997). This was further confirmed by the finding that urethelial derived NO inhibited contractility of the rat ureter (Mastrangelo et al. 2003). Since NOS expression could be detected in the nerve fibres of porcine intravesical ureter, an inhibitory effect on neurotransmission was hypothesised (Hernández et al. 1995). In addition, in human ureteral smooth muscles, the cGMP hydrolyzing PDE, PDE1, and PDE5 have been identified (Taher et al. 1994; Becker et al. 1998). The PDE1/5 inhibitor zaprinast, also the PDE5 inhibitor E4021, was able to relax human isolated ureteral tissues (Stief et al. 1995; Kühn et al. 2000). Recently, it was shown that the selective PDE5 inhibitors sildenafil, vardenafil, and tadalafil relaxed human ureteral tissue (Gratzke et al. 2007). These results from organ bath assay contractility studies are up to now not translated into animal models and also clinical data are missing. However, based on the previous findings, cGMP increasing agents might become an effective and safe treatment combination i.e. with analgesics for patients undergoing lithotripsy in the future.

9 Summary and Conclusions

The introduction of the nitrates for angina demonstrated for the first time, the importance of cGMP elevation for pharmacological intervention. However, it is established now that the NO/cGMP signaling cascade is a common mechanism regulating physiological functions throughout the whole organism, including all organs and tissues including the lower urinary tract. After the launch of PDE5 inhibitors for the treatment of erectile dysfunction, it become obvious that pharmacological interventions, increasing cGMP, in the corpus cavernosum have tremendous impact on penile function.

In the meantime, there is emerging evidence that the NO/cGMP system plays an important regulatory role in regulation of other lower urinary tract functions beyond penile tissue. Most importantly, approaches to increase cGMP with PDE5 inhibitors in bladders of LUTS patients resulted in a reduction of the bothersome symptoms in BPH patients. Therefore, PDE5 inhibitors might become a future treatment option for LUTS in BPH patients. In addition in OAB, first clinical results demonstrated that a cGMP increase could become a successful treatment option for bothersome urinary tract symptoms existing in both male and female patients.

The use of NO/cGMP interventions, especially also the use of PDE5 inhibitors, in other diseases like female sexual dysfunction, peyronies, premature ejaculation, and stone diseases is to date not as well characterized to draw final conclusions (Schwarz et al. 2007; Sandner et al. 2007). Since on the one hand, these diseases are hardly to treat until now, and on the other hand, safe and efficient compounds to increase

cGMP via PDE5 inhibition or sGC stimulation are available, future research and development efforts should try to establish the functional role of the NO/cGMP cascade within these diseases.

Combining the information about the role of NO/cGMP/PDE effects in the lower urinary tract together, the current available data clearly demonstrate that this pathway is a key component for lower urinary tract function.

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cGMP and cGMP-Dependent Protein Kinase in Platelets and Blood Cells

Ulrich Walter and Stepan Gambaryan

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Abstract Platelets are specialized adhesive cells that play a key role in normal and pathological hemostasis through their ability to rapidly adhere to subendothelial matrix proteins (platelet adhesion) and to other activated platelets (platelet aggregation). NO plays a crucial role in preventing platelet adhesion and aggregation. In platelets, cGMP synthesis is catalyzed by sGC, whereas PDE2, PDE3 and PDE5 are responsible for cGMP degradation. Stimulation of cGK by cGMP leads to phosphorylation of multiple target substrates. These substrates inhibit elevation of intracellular calcium, integrin activation, cytoskeletal reorganization, and platelet granule secretion, events normally associated with platelet activation. The NO/cGMP pathway also plays a significant role in many other blood cell types in addition to platelets. In leukocytes, depending on the specific cell type, cGMP signaling regulates gene expression, differentiation, migration, cytokine production, and apoptosis.

Keywords: Platelet · cGMP · cAMP · cGMP-dependent · Protein kinase · Soluble guanylyn cyclone

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Abbreviations

ABP	Actin binding protein
ADP	Adenosine diphosphate
AC	Adenylyl cyclase
ANP, BNP, and CNP	Atrial, brain and C-type natriuretic peptides respectively
cAK	cAMP-dependent protein kinase
cGK	cGMP-dependent protein kinase
GTP	Guanosine 5′-triphosphate
DC	Dendritic cells
GP1bβ	Glycoprotein 1bß
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
NO	Nitric oxide
IP_3	Inositol 1,4,5-trisphosphate
IRAG	IP_3 receptor associated cGK I substrate
NOS	Nitric oxide synthase
NFAT	Nuclear factor of activated T cells
MRP4	Nucleotide transporter multidrug resistance protein
pGCs	Particulate guanylyl cyclases
PDE	Phosphodiesterase
PG-I_2	Prostacyclin
PLC	Phospholipase C
PKC	Protein kinase C
RBC	Red blood cells
TCR	T cell receptor
sGC	Soluble guanylyl cyclase
VASP	Vasodilator-stimulated phosphoprotein
vWF	von Willebrand Factor

1 Introduction

Platelets play a key role in normal and pathological hemostasis through their ability to rapidly adhere to activated or injured endothelium and subendothelial matrix proteins (platelet adhesion) and to other activated platelets (platelet aggregation) (Ruggeri and Mendolicchio 2007). Platelets also play a pivotal role in cardiovascular diseases, and have emerged as a therapeutic target, since a wealth of evidence from large clinical trials supports that anti-platelet drugs have become paramount in the prevention and management of various diseases involving the cardiovascular, cerebrovascular, and peripheral arterial systems (Meadows and Bhatt 2007). Platelets are now considered key mediators of thrombosis, inflammation, and atherosclerosis (Libby 2002; Ross 1999). Moreover, very recent studies with murine models (Burger and Wagner 2003; Huo et al. 2003; Massberg et al. 2002) strongly support the hypothesis that activated platelets play an important triggering role even in the early phase of atherosclerosis (Gawaz et al. 2005).

A variety of factors, including collagen, fibrinogen, ADP, von Willebrand factor (vWF), thrombin, thromboxane, and others, promote platelet adhesion and aggregation by utilizing multiple intracellular signal transduction mechanisms. In addition to activating factors, in vivo circulating platelets are also continually exposed to inhibitory factors such as endothelium-derived nitric oxide (NO) and prostacyclin ($PG\text{-}I_2$) (Ruggeri and Mendolicchio 2007). Most of these activating and inhibitory factors bind to specific platelet receptors to stimulate signaling pathways which promote or inhibit platelet adhesion, aggregation, and secretion. A well-regulated equilibrium between these two opposing processes is thought to be essential for normal platelet and vascular function. An impairment of this equilibrium will promote either thrombotic or bleeding disorders.

This review will focus primarily on platelet inhibition by the intracellular second messengers cAMP and cGMP which play crucial roles in platelet inhibition (Fig. 1) (Gambaryan et al. 2004; Lohmann and Walter 2005; Munzel et al. 2003; Walter and Gambaryan 2004). cGMP and cAMP levels are increased in response to established

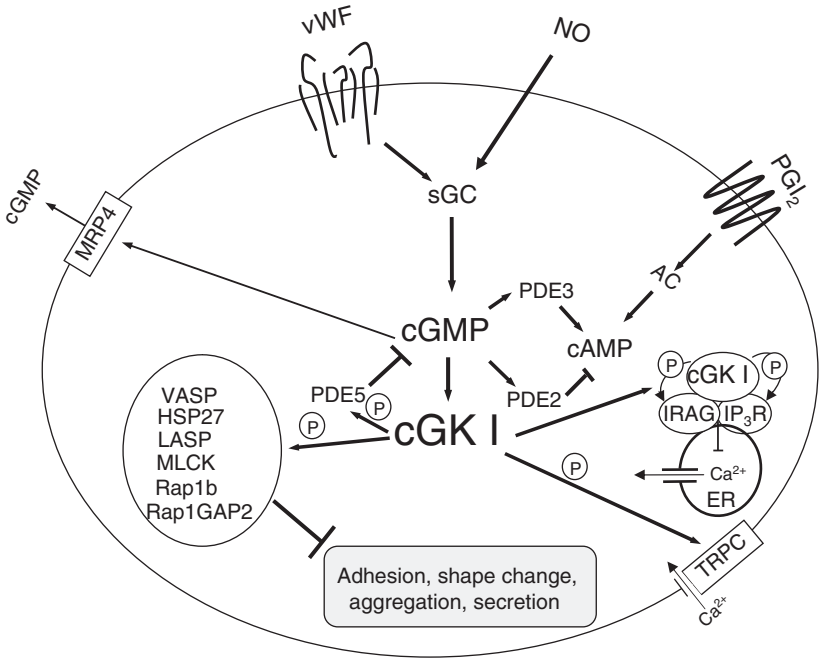

Fig. 1 cGMP/cGK I signaling in platelets. Intracellular cGMP concentration is regulated by synthesis (sGC) and degradation (mainly by PDE5, a well established cGK I substrate in platelets), as well as by nucleotide transporter multidrug resistance protein (MRP4)-mediated extrusion. cGMP also affects cAMP concentration by regulating the activities of PDE2 and PDE3. The major target of cGMP is cGK I which phosphorylates multiple substrates (VASP, HSP27, LASP, MLCK, Rap1b, Rap1GAP2, and TRPC) involved in different inhibitory pathways (adhesion, shape change, aggregation, and secretion) in platelets. In addition, cGK I forms a protein complex with and phosphorylates IP_3R and IRAG, thereby regulating calcium release from intracellular stores

platelet inhibitors such as NO and PG-I_2, respectively, which directly activate either soluble guanylyl cyclase (sGC) or G_s-protein-coupled prostacyclin receptors. Elevated cyclic nucleotide levels then activate the corresponding cGMP- and cAMP-dependent protein kinases (cGK and cAK, respectively), and stimulate phosphorylation of substrate proteins (Goldberg and Haddox 1977; Lohmann and Walter 2005; Munzel et al. 2003). For cGMP, other important intracellular platelet targets include the cGMP-stimulated phosphodiesterase 2 (PDE2), the cGMP-inhibited phosphodiesterase 3 (PDE3), and the cGMP-specific, cGMP-binding phosphodiesterase 5 (PDE5), which regulate platelet cAMP and cGMP levels and significantly contribute to important crosstalk between cAMP and cGMP signaling (Fig. 1) (Haslam et al. 1999; Schwarz et al. 2001). Elevation of either cAMP or cGMP mediates down-regulation of agonist-induced intracellular calcium signaling, fibrinogen binding, adhesion, and aggregation of human platelets (Munzel et al. 2003; Schwarz et al. 2001). Although there is considerable cross-talk between cAMP and cGMP signaling events, studies with cGK-deficient human and murine platelets strongly supported the conclusion that many cGMP-mediated effects in these two cell types are mediated by cGK I (Eigenthaler et al. 1993; Massberg et al. 1999).

The NO/cGMP pathway also plays a significant role in many other blood cell types, in addition to platelets. In leukocytes, depending on the cell type, cGMP signaling regulates gene expression, differentiation, migration, cytokine production, and apoptosis. However, functions of cGMP and especially cGKs identified in leukocytes in vitro using primarily pharmacological inhibitors and stimulators may need further study, since cGK expression has been shown to be strongly down regulated even in short-term cultures of, for example, T lymphocytes (Fischer et al. 2001).

2 Platelets and cGMP

2.1 Regulation of Platelet cGMP Synthesis

In general, the cellular level of cGMP is controlled by synthesis via particulate guanylyl cyclases (pGCs) and/or soluble guanylyl cyclase (sGC), and by degradation via phosphodiesterases (Sect. 2.2). pGCs (GC-A, GC-B, and GC-C) are receptors for the family of natriuretic peptides (NPs) consisting of atrial, brain and C-type natriuretic peptides (ANP, BNP, and CNP, respectively). ANP and BNP are ligands for GC-A, CNP for GC-B. The GC-C receptor, mainly expressed in intestinal epithelium, binds heat-stable enterotoxins, guanylin, and uroguanylin (Feil et al. 2003; Kuhn 2003). However, unlike other cell types, platelets appear not to express any type of pGC. Instead platelet cGMP synthesis is regulated by sGC, the most sensitive physiological receptor for NO. NO is formed from L-arginine by nitric oxide synthase (NOS) which consists of the three isoforms, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), encoded by distinct genes (Davis et al. 2001).

NO, in addition to its major effect on activation of sGC, also modulates protein functions by S-nitrosylation of cysteine or nitration of tyrosine residues (Davis et al. 2001). However, experiments with sGC-deficient mice clearly established that most, if not all, inhibitory effects of NO on platelets are mediated by the sGC/cGMP/cGK system (Friebe et al. 2007).

Endothelial cells, which express a high amount of constitutively active eNOS, are the major source of NO in plasma. In addition, eNOS and iNOS have been described to be expressed in platelets, however, data concerning expression, regulation, and function of eNOS and iNOS in platelets are highly controversial (Gkaliagkousi et al. 2007; Naseem and Riba 2008). In contrast to these publications, our data (Gambaryan et al. 2008) and that of others (Ozuyaman et al. 2005) clearly demonstrated that human and mouse platelets do not express any functionally active NOS protein, indicating that endothelial cell-derived NO is the major activator of platelet sGC.

sGC is formed by dimerization of either an α_1- or α_2-subunit to the β1-subunit, and these dimers have indistinguishable enzymatic activity (Russwurm and Koesling 2002). In platelets, only the α_1 and β_1 subunits of sGC are expressed (Mergia et al. 2006). Binding of NO to the heme moiety of the cyclase induces its capacity to synthesize cGMP. However, activation of sGC does not necessarily require NO, since in some cases, NO-independent regulation of sGC activity occurs by protein-protein interaction of sGC with Hsp70, Hsp90, and PSD95, as well as by Ser/Thr and/or Tyr phosphorylation of sGC (Balashova et al. 2005; Louis et al. 1993; Meurer et al. 2005). Recently a new mechanism of NO-independent tyrosine phosphorylation of the sGC β-subunit and activation of sGC was described in platelets stimulated with vWF (Gambaryan et al. 2008).

2.2 Regulation of Platelet cGMP Degradation

Hydrolysis of platelet cGMP is catalyzed by phosphodiesterases (PDEs), a large group of enzymes consisting of at least 11 different families (Bender and Beavo 2006a). PDEs hydrolyze the 3′-phosphoester bond of cyclic nucleotides, converting them into biologically inactive 5′-nucleotide metabolites. At present, only three PDEs have definitely been shown to be expressed in platelets, cGMP-stimulated PDE2, cGMP-inhibited PDE3, and the cGMP-binding, cGMP-specific PDE5 (Haslam et al. 1999). PDE2 hydrolyzes both cGMP and cAMP with similar affinities and is stimulated by binding of cGMP to regulatory sites. PDE3 preferentially hydrolyzes cAMP and is inhibited by binding of cGMP. In platelets, depending on the experimental conditions and species (human, mouse, rabbit, or bovine), binding of cGMP to PDE2 or PDE3 can significantly influence cAMP levels (reviewed in (Haslam et al. 1999). PDE5 is activated by cGMP and is highly specific for cGMP hydrolysis, having a *Km* of 1 μM for cGMP and 150 μM for cAMP (Schwarz et al. 2001). Pharmacological interest in PDE5 increased once it becomes obvious that PDE5 inhibitors improve the symptoms of male erectile dysfunction

(Rotella 2002). Human platelets contain a high concentration of PDE5, and several reports described that PDE5 inhibitors, to some extent, suppress agonist-induced platelet aggregation (Halcox et al. 2002; Wallis et al. 1999). This might, in some cases, have in vivo significance since there are case reports of hemorrhoidal and acute variceal bleeding after using PDE5 inhibitors (Sheikh et al. 2001; Tzathas et al. 2002). However, others (Dunkern and Hatzelmann 2005; Levin et al. 1982) showed that PDE5 inhibitors only strongly potentiated inhibitory effects of endogenous NO without themselves having direct inhibitory effects on platelet activation. In addition to cGMP–degrading PDEs, ATP-dependent cGMP transport by the nucleotide transporter multidrug resistance protein (MRP4), highly expressed in platelet dense granules and plasma membrane, may significantly contribute to extrusion and regulation of intracellular platelet cGMP (Jedlitschky et al. 2004). However, the functional significance of cGMP transport by MRP4 for platelet activation/inhibition remains to be established.

2.3 Platelet cGMP Effectors Systems, Platelet cGK

cGMP effects can be mediated by a number of effectors including cGMP-gated channels, cGMP-regulated phosphodiesterases, cGKs, and possibly by cAK at high cGMP concentrations. In platelets, cGMP effects are mediated predominantly by cGMP-PDEs (Sect. 2.2) and cGKs. Mammalian cGK exists in two major forms, cGK I and cGK II, which have been cloned and defined as products of two separate genes (reviewed in Eigenthaler et al. 1999; Lohmann and Walter 2005). Human platelets express only cGK Iβ, whereas mouse platelets additionally express a small amount of cGK Iα (Antl et al. 2007). The cGK I concentration in human platelets (3.65 μM holoenzyme, equivalent to 14.6 μM cGMP-binding sites) is higher than that in any other cell type examined (Eigenthaler et al. 1992). The important role of cGK I inhibition of platelet activation in vitro and in vivo has been conclusively demonstrated in cGK I-deficient (cGK I KO) murine platelets (Massberg et al. 1999). In vitro activation of platelet cGK I by membrane-permeable cGMP analogs and NO donors inhibited agonist-induced serotonin release, shape change, and aggregation in wild-type platelets, but not cGK I KO mouse platelets. In cGK I KO mouse platelets, expression and functional activity of cAK is not altered, and there is also no cross-activation of cAK by cGMP. The reverse, activation of cGK I by cAMP seems also not to occur in platelets, indicating that the cAMP and cGMP signaling cascades inhibit platelet activation independently of each other (Massberg et al. 1999). In vivo studies using cGK I KO mice showed that platelet cGK I, but not endothelial or smooth muscle cGK I, is essential to prevent intravascular adhesion and aggregation of platelets after ischemia, and a defect due to cGK loss was not compensated by the cAMP/cAK system (Massberg et al. 1999).

cGK I activation in platelets inhibits several agonist-induced events including the increase in intracellular calcium levels, integrin activation, cytoskeletal reorganization, and platelet granule secretion (Fig. 1). Cytosolic calcium levels are elevated in

platelets in response to most platelet agonists which activate different isoforms of phospholipase C (PLC) to stimulate inositol 1,4,5-trisphosphate (IP_3)-mediated release of Ca^{2+} from intracellular calcium stores and subsequent store-operated Ca^{2+} entry (Geiger et al. 1992; Geiger et al. 1994). Activation of cGK I by NO donors or membrane-permeable cGMP analogs strongly inhibited agonist-evoked calcium mobilization from intracellular stores (Fig. 1) and calcium entry via store-operated calcium channels, but not via ADP-activated calcium channels (Geiger et al. 1994). Recently it was shown that signaling by IP_3 receptor associated cGK I substrate protein (IRAG) is a major mediator of cGMP-dependent inhibition of agonist-induced increase in intracellular calcium level and platelet aggregation. In vivo, activation of cGK I abolished arterial thrombus formation after vascular injury in wild type mice, but not IRAG deletion mutant mice (Antl et al. 2007).

Reorganization of platelet cytoskeletal proteins, and actin filament turnover, play significant roles in platelet function and formation of the integrin signaling complex. The most abundant platelet surface integrin, $\alpha_{IIb}\beta_3$, exists in a low-affinity state unable to bind soluble ligands in unstimulated platelets. Agonist-generated inside-out signals are required to induce conformational changes and clustering that enable integrin $\alpha_{IIb}\beta_3$ to bind soluble fibrinogen and activate platelets. Integrin activation involves integrin oligomerization, and coordinated activities of protein tyrosine kinases and phosphatases which activate the Src family of kinases and create an integrin-based signaling complex (Kasirer-Friede et al. 2007). Integrin signaling is inhibited by the cytoskeletal-associated vasodilator-stimulated phosphoprotein (VASP), a major cGK substrate in platelets (Halbrugge et al. 1990; Reinhard et al. 2001). Platelets from VASP-deficient mice exhibit enhanced agonist-induced activation of surface P-selectin expression and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ (Aszodi et al. 1999; Hauser et al. 1999). VASP also negatively regulates platelet adhesion to the vascular wall in vivo; NO/cGMP/cGK-dependent inhibition of agonist-induced platelet adhesion is defective in VASP-deficient mice (Massberg et al. 2004).

Platelet secretion, in which dense granules, α-granules, and lysosomes are released, is a very complex process regulated by multiple intracellular signaling systems. Cytoskeletal protein reorganization, increase of cytosolic Ca^{2+} concentration, and activation of protein kinase C (PKC) are essential for platelet secretion (Elzagallaai et al. 2001). Activation of cGK I inhibits platelet secretion by reducing both cytosolic Ca^{2+} concentration and PLC-dependent activation of PKC (Schwarz et al. 2001). However, the detailed molecular mechanisms of NO/cGMP/cGK I inhibition of platelet secretion require further elucidation.

2.4 cGK Substrates and Their Functions in Platelets

The cytoskeleton-associated protein VASP was initially isolated from human platelets and characterized as a prominent substrate of both cAK and cGK (Halbrugge et al. 1990; Halbrugge and Walter 1989; Walter et al. 1993). VASP

is phosphorylated in vitro and in intact human platelets, at Ser^{157} and Ser^{239} by both cAK and cGK. VASP is phosphorylated preferentially at Ser^{239} by cGK, and at Ser^{157} by cAK, the latter causing a mobility shift in VASP Mr from 46 to 50 kDa on SDS-polyacrylamide gels. Phosphorylation affects the intracellular localization of VASP, and impairs VASP interaction with certain SH3-domain proteins and F-actin (Benz et al. 2008). VASP phosphorylation also correlates with inhibition of both $\alpha_{IIb}\beta_3$ integrin and platelet activation (Horstrup et al. 1994). Several other platelet proteins involved in cytoskeletal reorganization are also cGK substrates, e.g. myosin light chain kinase (MLCK) (Nishikawa et al. 1984), heat shock protein 27 (Hsp27) (Butt et al. 2001), LIM and SH3 domain protein (LASP) (Butt et al. 2003), Rap1b (Danielewski et al. 2005), and Rap1GAP2 (Schultess et al. 2005) (Table 1). Phosphorylation of Hsp27 is probably involved

Table 1 Identified cGK substrates in platelets

Protein	Size (kDa)	Phosphorylation site	Proposed function
VASP	46/50	S^{239}, S^{157}, T^{278}	Inhibition of VASP binding to F-actin and SH3 proteins, resulting in decreased adhesion (Massberg et al. 2004, Benz et al. 2008)
LASP	28	S^{146}	Function in platelets is unknown (Butt et al. 2003)
Heat shock protein 27 (HSP 27)	27	T^{143}, S^{82}	Reduction of MAP kinase-stimulated actin polymerization (Butt et al. 2001)
IP_3-receptor	240	S^{1755}, S^{1589}	Inhibition of calcium release from intracellular stores (El-Daher et al. 2000)
IP_3-receptor associated cGK substrate (IRAG)	125	S^{696}, S^{683}	Inhibition of calcium release from intracellular stores (Antl et al. 2007)
Phosphodiesterase 5 (PDE5)	92	S^{92}	Enhanced cGMP degradation (Haslam et al. 1999)
Transient receptor potential canonical protein (TRPC1, TRPC6)	102	T^{70}, S^{322} (TRPC6)	Inhibiton of calcium transient (Hassock et al. 2002)
Myosin light chain kinase (MLCK)	98	Unknown	Decreased catalytic activity (Nishikawa et al. 1984)
Rap 1b	21	S^{179}	Inhibition of Rap 1b activation (Danielewski et al. 2005)
Rap1GAP2	76	S^{7}	Disruption of the complex of Rap1GAP2 with 14-3-3 protein (Hoffmeister et al. 2008)

in the reduction of MAP kinase-stimulated actin polymerization (Butt et al. 2001). LASP is a membrane-associated scaffolding and actin-binding protein which upon phosphorylation changes its distribution and actin-binding properties. However, the functional consequences of LASP phosphorylation in platelets are unknown (Butt et al. 2003). Rap1b is required for normal integrin $\alpha_{IIb}\beta_3$ signalling in platelets (Chrzanowska-Wodnicka et al. 2005), and inhibition of integrin $\alpha_{IIb}\beta_3$ activity by cGK is, at least partly, connected with cGK-induced phosphorylation of Rap 1b (Danielewski et al. 2005). GTPase-activating protein of Rap 1(Rap1GAP2) was also recently identified as a cGK substrate in platelets (Schultess et al. 2005). Although phosphorylation of Rap1GAP2 by cGK does not impact directly on the catalytic activity of Rap1GAP2, it does disrupt the complex of Rap1GAP2 with 14-3-3 protein, reversing the 14-3-3 attenuation of Rap1GAP2-mediated inhibition of cell adhesion, which might contribute to the inhibition of thrombus formation by endothelium-derived NO (Hoffmeister et al. 2008).

At least three key proteins involved in the regulation of intracellular Ca^{2+} concentration, the IP_3 receptor (El-Daher et al. 2000; Murthy and Zhou 2003), the IP_3 receptor-associated cGK I substrate (IRAG) (Schlossmann et al. 2000), and the transient receptor potential canonical (TRPC) proteins (Hassock et al. 2002) have been described as substrates of cGK in platelets (Table 1). All of the three known IP_3 receptors (IP_3R-I, IP_3R-II, and IP_3R-III) contain cGK phosphorylation consensus sites and are expressed in platelets. IP_3R-I is predominantly localized in intracellular membranes, whereas IP_3R-II, and IP_3R-III are more associated with the platelet plasma membrane (El-Daher et al. 2000). IP_3R-I in smooth muscle cells is phosphorylated by cGK at Ser^{1755} (Murthy and Zhou 2003), however the functional consequences of IP_3R phosphorylation by cGK are unclear, and whether IP_3R is phosphorylated by cGK in platelets at all is still debated (El-Daher et al. 2000). The cGK I substrate, IRAG, has been identified in a complex with IP_3R-I and cGK I (Schlossmann et al. 2000). Phosphorylation of IRAG by cGK at Ser^{696} (human) and Ser^{677} (mouse) is responsible for cGK I-induced inhibition of IP_3-stimulated calcium release from intracellular calcium stores (Antl et al. 2007).

Of the TRPC protein family, TRPC6 and TRPC1 have been conclusively shown to be expressed in platelets, however it is still likely that other members will be found. TRPC6 was found exclusively in the plasma membrane fraction, whereas TRPC1 was detected predominantly in intracellular membranes (Authi et al. 2007). Both TRPC6 and TRPC1 have consensus sites for phosphorylation by cGK and cAK and can be phosphorylated in intact platelets by both kinases, however the direct consequence of this phosphorylation is unclear (Authi et al. 2007; Hassock et al. 2002)

Several other cGK substrates, including RhoA (Sawada et al. 2001), Pak1 (Fryer et al. 2006), phospholipase A_2 (PLA_2) (Murthy and Makhlouf 1998), the thromboxane receptor (TXA_2) (Yamamoto et al. 2001), and the Ca^{2+}-activated K^+ channel (BKca) (Fukao et al. 1999), have been identified in platelets, but the functional consequences of their phosphorylation by cGK remain to be elucidated.

cGK and cAK have overlapping substrate specificities, and in many cases phosphorylate proteins on the same site. Such common substrates in platelets are VASP,

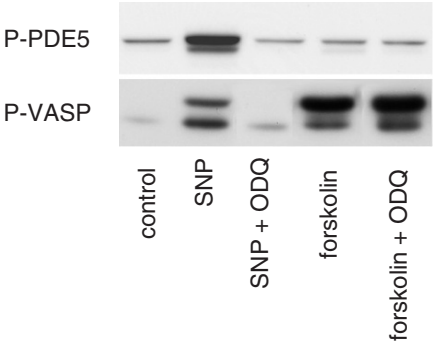

Fig. 2 PDE5 in platelets is a specific cGK I substrate. Washed human platelets were incubated with an NO-donor (SNP, 10 µM, 2 min) or AC activator (forskolin, 5 µM, 2 min), and in some cases, preincubated with an sGC inhibitor (ODQ, 5 µM, 5 min). PDE5 is phosphorylated only by cGK I, whereas VASP is phosphorylated by both cAK and cGK kinases

LASP, Hsp27, Rap1, Rap1GAP2, and others. Exceptions include IRAG, which forms a complex with cGK I and is probably phosphorylated only by cGK I (Schlossmann et al. 2000), and PDE5, which in platelets is phosphorylated exclusively by cGK and not by cAK (Fig. 2). Several established cAK substrates strongly involved in platelet inhibitory pathways, including actin binding protein (ABP) (Chen and Stracher 1989), caldesmon (Hettasch and Sellers 1991), $G\alpha_{13}$ (Manganello et al. 1999), and β-subunit of glycoprotein 1b (GP1bβ) (Bodnar et al. 2002; Wardell et al. 1989), can potentially also be phosphorylated by cGK, however this phosphorylation and its functional consequences remain to be elucidated. A recently developed phosphoproteomic approach for identification of platelet phosphorylation sites revealed new, putative cGK and cAK substrates in platelets (Zahedi et al. 2008), indicating that the list of cGK substrates in platelets will certainly be expanded.

3 cGMP and cGMP Effector Systems in Other Blood Cell Types

In addition to platelets, the NO/cGMP pathway was found to play a significant role in virtually all other blood cells. Recently the expression of eNOS and the functional relevance of red blood cell (RBC)-derived NO was described (Kleinbongard et al. 2006). The expression of sGC in RBC has not been conclusively demonstrated, however sGC activity was shown to increase in RBC from patients with sickle cell disease (Conran et al. 2004). The presence of cGMP effector systems in RBC is currently unclear, as there is no data in the available literature concerning expression and function of cGMP-regulated PDEs or cGKs in RBC.

In leukocytes, depending on the cell type, the cGMP signaling system plays a significant role in gene expression, differentiation, migration, cytokine production,

and apoptosis. Dendritic cells (DC) play a pivotal role in initiating and instructing adaptive immune responses, by activating native T cells in peripheral lymphoid tissues. Upon activation, peripheral DC migrate to T cell areas in the lymph nodes. In lipopolysaccharide (LPS)- and NO donor- stimulated DC, activation of sGC and increase of cGMP correlate with strong up regulation of cGK Iβ expression, and cGK Iβ-dependent phosphorylation of VASP is involved in regulation of DC migration toward the lymph-node-directing chemokine, CCL19 (Giordano et al. 2003; Giordano et al. 2006). In addition to sGC, DC were shown to express the ANP receptor, GC-A, which responds to ANP with an increase in intracellular cGMP. ANP treatment changes the pattern of cytokine production of LPS-stimulated DC, and these DC polarize native $CD4^+$ T cells toward a Th2 phenotype (Morita et al. 2003).

Human T lymphocytes express cGK Iβ which mediates inhibitory effects of cGMP on cell proliferation and interleukin 2 production (Fischer et al. 2001). In human T lymphocytes, stimulation of cGK by NO donors or membrane-permeable cGMP analogs strongly stimulated p42-44 and p38 MAP kinases (Fischer et al. 2001), whereas in platelets, stimulation of cGK I had no significant effect on basal mitogen-activated protein kinases (MAPK) activity, and inhibited agonist-induced activation of MAP kinases (Begonja et al. 2007; Schwarz et al. 2000). cGK I also has different effects on intracellular calcium concentration in platelets versus lymphocytes. In platelets, cGK I activation decreases agonist-induced intracellular calcium concentration (Geiger et al. 1992), whereas in mouse T lymphocytes, stimulation of the T cell receptor (TCR) by anti-TCR antibody NO-independently stimulated sGC, cGMP, and cGK I to increase intracellular calcium. Elevated intracellular calcium promoted dephosphorylation and nuclear translocation of the nuclear factor of activated T cells (NFAT) and subsequent gene expression (Gomes et al. 2006).

Macrophages can be derived from monocytes that normally circulate in peripheral blood. In response to cytokines, monocytes leave the circulation and enter a tissue where they can differentiate into macrophages (Morrissette et al. 1999). PDE expression pattern changes in response to cytokine-stimulation alter the intracellular concentrations of cAMP and cGMP and play an essential role in monocyte differentiation (Bender and Beavo 2006b; Bender et al. 2005). Also during monocyte differentiation into macrophages, the expression of sGC decreases, and that of GC-A increases (Bender et al. 2004). cGMP has many effects on monocyte/macrophages functions which seem controversial. It has been reported that cGMP can inhibit iNOS induction (Kiemer and Vollmar 1998), stimulate TNF-α release (Tamion et al. 1999), and stimulate (Vollmar et al. 1997) or inhibit (Mattana and Singhal 1993) phagocytosis. Data concerning functions of cGMP and especially cGKs in leukocytes, acquired using only pharmacological inhibitors and stimulators, should be considered with caution, since the expression of cGKs, as shown for T lymphocytes (Fischer et al. 2001), may be strongly down regulated even in short-term cultures.

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cGMP Signalling in the Mammalian Brain: Role in Synaptic Plasticity and Behaviour

Thomas Kleppisch and Robert Feil

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Abstract The second messenger cyclic guanosine $3',5'$-monophosphate (cGMP) plays a crucial role in the control of cardiovascular and gastrointestinal homeostastis, but its effects on neuronal functions are less established. This review summarizes recent biochemical and functional data on the role of the cGMP signalling pathway in the mammalian brain, with a focus on the regulation of synaptic plasticity, learning, and other complex behaviours. Expression profiling, along with pharmacological and genetic manipulations, indicates important functions of nitric oxide (NO)-sensitive soluble guanylyl cyclases (sGCs), cGMP-dependent protein kinases (cGKs), and cGMP-regulated phosphodiesterases (PDEs) as generators, effectors, and modulators of cGMP signals in the brain, respectively. In addition, neuronal cGMP signalling can be transmitted through cyclic nucleotide-gated (CNG) or hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels. The

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canonical NO/sGC/cGMP/cGK pathway modulates long-term changes of synaptic activity in the hippocampus, amygdala, cerebellum, and other brain regions, and contributes to distinct forms of learning and memory, such as fear conditioning, motor adaptation, and object recognition. Behavioural studies indicate that cGMP signalling is also involved in anxiety, addiction, and the pathogenesis of depression and schizophrenia. At the molecular level, different cGK isoforms appear to mediate effects of cGMP on presynaptic transmitter release and postsynaptic functions. The cGKs have been suggested to modulate cytoskeletal organization, vesicle and AMPA receptor trafficking, and gene expression via phosphorylation of various substrates including VASP, RhoA, RGS2, hSERT, GluR1, G-substrate, and DARPP-32. These and other components of the cGMP signalling cascade may be attractive new targets for the treatment of cognitive impairment, drug abuse, and psychiatric disorders.

1 Introduction

The cyclic nucleotide cyclic guanosine $3',5'$-monophosphate (cGMP) is an important second messenger in the cardiovascular and gastrointestinal system, where it controls cellular functions ranging from growth to contractility (Beavo and Brunton 2002). However, its function in the nervous system is not so well studied. Cyclic GMP is generated in response to natriuretic peptides and nitric oxide (NO) from membrane-bound particulate guanylyl cyclases (pGCs) and cytosolic soluble guanylyl cyclases (sGC), respectively (see the chapters by Kuhn et al., Marletta et al. and Koesling et al., this volume). The effects of cGMP are transduced via a number of distinct biochemical pathways. Cyclic GMP effectors include cGMP-dependent protein kinases (cGKs), cGMP-modulated cation channels, and cGMP-regulated phosphodiesterases (PDEs) that hydrolyze cyclic nucleotides (see the chapters by Hofmann et al., Biel et al. and Menniti et al. this volume). The past several years have seen a renewed interest in cGMP research with the discovery of novel cGMP-dependent mechanisms and functions and with the realization of the therapeutic potential of targeting the cGMP pathway, particularly in the treatment of erectile dysfunction and cardiovascular disorders (Feil and Kemp-Harper 2006; Kemp-Harper and Feil 2008). In this review, we will summarize recent developments in the field of neuronal cGMP signalling, with a focus on the regulation of synaptic plasticity and behaviour in mammals. Other aspects of cGMP signal transduction in the central and peripheral nervous system have been covered in the recent literature, for example, the role of NO, cGMP, and cGKs in neuroprotection and neurodegeneration (Calabrese et al. 2007), in axon guidance of sensory neurons and nociception, and in circadian rhythms (Feil et al. 2005a).

Our knowledge of the in vivo significance of cGMP signalling is mainly based on pharmacological or genetic manipulation of its components. Drugs that elevate cGMP levels include inhibitors of cGMP-hydrolyzing PDEs (e.g., the PDE5 inhibitor, sildenafil) (Menniti et al. 2006) and NO-independent activators of sGC

(Evgenov et al. 2006). To date, genetically modified mouse models are available for most of the cGMP generators and effectors (Hofmann et al. 2004). Among the mouse lines that have been used in the analysis of brain functions are conventional sGC knockout mice (Buys et al. 2008; Friebe et al. 2007; Mergia et al. 2006) and various mouse mutants lacking cGKs in the whole nervous system (Pfeifer et al. 1996, 1998; Weber et al. 2007; Wegener et al. 2002) or selectively in specific areas of the brain (Feil et al. 2003; Kleppisch et al. 2003). It is important to note that both genetic as well as pharmacological strategies can produce phenotypes unrelated to the protein or brain region of interest. Furthermore, phenotypes, particularly behavioural ones, can be strongly affected by the genetic background and environment of the animals (Siuciak et al. 2008). Thus, the reader should keep in mind that genetic modifications and drug treatments (van Staveren et al. 2005) may produce artefacts and controversial phenotypes, and that results obtained in rodent models cannot always be transferred to humans.

2 Components of cGMP Signalling and Their Expression in the CNS

Cyclic GMP can be generated by pGCs that are stimulated by a family of natriuretic peptides (Kuhn 2003) or by NO-sensitive sGCs (Friebe and Koesling 2003). Natriuretic peptides and their receptors have been found in the brain (DiCicco-Bloom et al. 2004; Herman et al. 1993; Komatsu et al. 1991; Sudoh et al. 1988, 1990), but their functions in the CNS remain largely unknown and, therefore, they will not be discussed further. It is also noteworthy that NO is a highly reactive radical, which tends to react with other molecules, resulting in the oxidation, nitrosylation or nitration of cellular proteins with concomitant effects on neuronal function in a cGMP-*independent* manner (Guix et al. 2005). On the other hand, there is a large body of evidence that many NO effects in the brain are mediated via stimulation of sGCs and elevation of cGMP. The mammalian sGC is a heterodimeric hemoprotein that exists in two isoforms, $\alpha_1\beta_1$ and $\alpha_2\beta_1$, which appear to have similar regulatory and enzymatic properties. Both sGC isoforms are expressed in the brain. The analysis of NO-stimulated cGMP synthesis (De Vente et al. 1998) and sGC subunit expression by RNA in situ hybridization and Western blotting (Gibb and Garthwaite 2001; Mergia et al. 2003) indicated the presence of at least one sGC isoform in most areas of the brain including the hippocampus and cerebellum. However, immunohistochemical studies on the precise distribution of sGC proteins at the cellular level are relatively rare and largely confined to the hippocampus (Burette et al. 2002; Szabadits et al. 2007). The C-terminus of the α_2 subunit allows a specific interaction with the synaptic adaptor protein PSD-95 (postsynaptic density-95) suggesting a localization of the $\alpha_2\beta_1$ heterodimer at postsynaptic membranes (Russwurm et al. 2001).

Principally, the brain expresses all the known cGMP target proteins, such as cGMP-regulated cation channels (for further discussion, see also the chapter by Biel et al., this volume), cGMP-binding PDEs and cGKs. The mammalian PDE

superfamily consists of 11 gene families comprising at least 21 genes that encode perhaps more than 50 protein variants (Conti and Beavo 2007). All PDEs have significant similarity in their catalytic domain but differ in their regulatory regions. Among the eight cGMP-degrading PDEs, several "simply" hydrolyze cGMP (e.g., cGMP-PDE9) or both cGMP and cAMP (e.g., cGMP/cAMP-PDE10 and cGMP/cAMP-PDE11), whereas others are also modulated by cGMP binding to allosteric sites (e.g., cGMP-stimulated cGMP-PDE5, the target of sildenafil). Interestingly, some cGMP-regulated PDEs hydrolyze cGMP and/or cAMP (e.g., cGMP-stimulated cAMP/cGMP-PDE2 and cGMP-inhibited cAMP-PDE3) allowing for cross-talk between cGMP and cAMP signalling. Hence, PDEs are increasingly recognized as important modulators of the spatiotemporal dynamics of cyclic nucleotide signals and of the formation of specific cAMP/cGMP microdomains in the cell. Virtually all the PDEs are expressed in the CNS, making them attractive targets for drug development (Menniti et al. 2006). Indeed, pharmacological inhibitors are available for most PDEs (e.g., milrinone for PDE3, sildenafil for PDE5, and Bay 73–6691 for PDE9). The individual PDEs are found in distinct regions of the CNS (e.g., PDE3 in the striatum and hippocampus, PDE5 in the spinal cord and cerebellum, and PDE9 throughout the brain). In some types of neurons two or more PDEs are coexpressed but localize to different subcellular compartments, in line with their potential role in the generation and maintenance of cyclic nucleotide compartments.

The cGKs belong to the AGC family of Ser/Thr protein kinases and convert cGMP elevations into alterations of cellular functions via phosphorylation of substrate proteins (Hofmann et al. 2006). Mammals have two cGK genes that generate three cGK proteins; the *prkg2* gene encodes the cGK type II (cGKII) and the *prkg1* gene encodes, by use of alternative promoters and 5′ exons, two isoforms of the cGK type I (cGKI), cGKIα and cGKIβ. These isoforms differ only in their N-terminal domains that are involved in homodimerization, cGMP-regulation of kinase activity (the Iα isoform being ≈10-fold more sensitive to cGMP activation than the Iβ isoform), and specific interactions with substrates and other proteins. While the cGKI isozymes are predominantly found in the cytosol, the cGKII is myristoylated and, thereby, anchored to the plasma membrane. All cGKs are expressed in the nervous system. In contrast to cGKI antibodies, the availability of suitable antibodies against cGKII is limited and, therefore, cGKII expression has been mainly studied at the mRNA level. Previous studies suggested that cGKII is widely distributed in the mammalian brain and that cGKI expression is more restricted (Feil et al. 2005a). However, a comprehensive analysis of mRNA expression in the mouse brain (Lein et al. 2007) (http://brain-map.org/welcome.do) as well as recent immunohistochemical data (Feil et al. 2005b) support the view that the cGKI is the predominant isoform in the CNS. The latter study confirmed the expression of cGKI protein in previously described areas such as cerebellar Purkinje cells (Lohmann et al. 1981), hippocampus (Kleppisch et al. 1999), suprachiasmatic nucleus (Revermann et al. 2002), and dorsomedial hypothalamus (El-Husseini et al. 1999), and identified a number of additional cGKI-positive regions, such as medulla, subcommissural organ, cerebral cortex, amygdala, habenulae, various hypothalamic regions, olfactory bulb, pituitary gland, and the ganglion cell layer of the retina. RNA in

situ hybridization and immunoblotting with isoform-specific probes (Geiselhoringer et al. 2004) and antibodies (Feil et al. 2005b), respectively, indicated that the cGKIα isoform is predominant in the cerebellum and medulla, whereas the cGKIβ isoform is prominent in the cortex, hippocampus, hypothalamus, and olfactory bulb. Similar levels of the isoforms were detected in the pituitary gland and eye. Thus, it appears that distinct brain regions express distinct cGKs that signal via distinct pathways.

Taken together, the expression pattern of the individual components of the cGMP signalling system strongly supports the functional role of the canonical NO/sGC/cGMP/cGK pathway in the brain, and of PDEs as important enzymes for shaping cyclic nucleotide signals and for converting cGMP into cAMP signals and vice versa.

3 Role of cGMP Signalling in Synaptic Plasticity and Behaviour Related to Various Functional Systems of the CNS

Formation and retrieval of memories are fundamental processes highly relevant for adaptive behaviour. More than half a century ago, Donald Hebb (1949) proposed that learning and memory are based on long-lasting activity-dependent changes of synaptic transmission in a neural network. Meanwhile, so-called Hebbian forms of synaptic plasticity have been observed in many brain regions and types of neurons (Citri and Malenka 2007; Malenka and Bear 2004). Moreover, compelling evidence links two forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) of synaptic transmission, to various types of learning (Ito 2002; Manahan-Vaughan and Braunewell 1999; McKernan and Shinnick-Gallagher 1997; Rogan et al. 1997; Sigurdsson et al. 2007; Whitlock et al. 2006). Mechanisms similar to LTP also appear to play a major role in long-lasting modulation of responses to sensory and somatosensory stimuli after repetitive challenge, e.g., long-term sensitization of nociception. The following paragraphs summarize the role of cGMP signalling in these phenomena in major functional systems and regions of the CNS.

3.1 Hippocampus

The hippocampal formation serves an important function for so-called declarative memory, i.e., the memory of facts and events (Milner 2003; Scoville and Milner 1957). Since the first observation of LTP in the perforant pathway of the hippocampus (Bliss and Lomo 1973), this structure in the medial temporal lobe became the focus of intense research aimed at resolving molecular mechanisms of synaptic plasticity.

Not surprisingly, cGMP soon got into the picture as an intracellular second messenger that could mediate the effect of NO in *N*-methyl-D-aspartic acid (NMDA) receptor-dependent LTP of Schaffer collateral/CA1 synapses in the hippocampus.

This form of synaptic plasticity has been characterized in great detail (for review see Lisman and Raghavachari 2006; Malenka and Bear 2004). According to a current scheme (Fig. 1), NMDA receptor-mediated Ca^{2+}-influx activates a Ca^{2+}/calmodulin-activated neuronal NO synthase (nNOS) leading to NO synthesis in the postsynapse. Subsequently, NO diffuses as a retrograde messenger across the synaptic cleft to activate its effectors in the presynaptic terminal (Arancio et al. 1996; Garthwaite and Boulton 1995; Hawkins et al. 1998; Huang 1997). The bulk of pharmacological studies and the analysis of transgenic mouse models (Table 1)

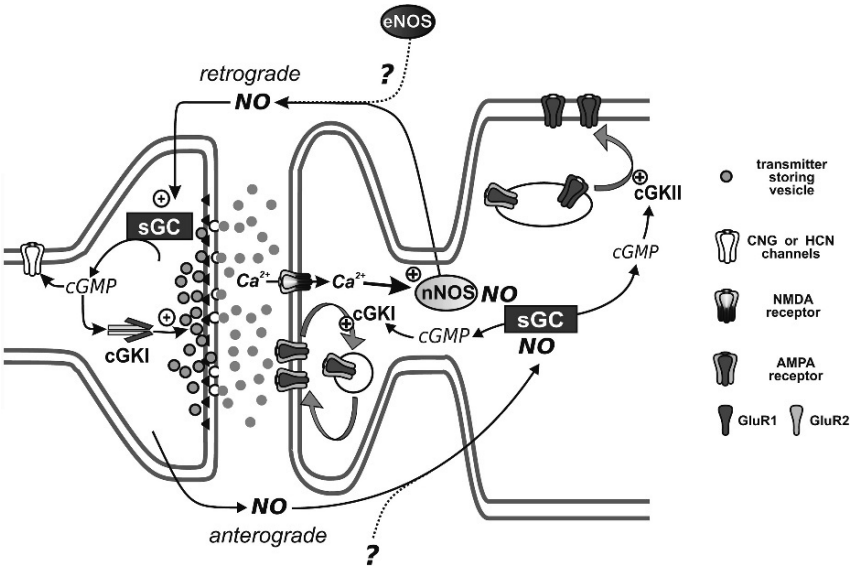

Fig. 1 Current concepts of cGMP signalling in synaptic plasticity. cGMP signals are initiated by NO through activation of sGC. NO is generated by a Ca^{2+}/calmodulin-regulated nNOS. Depending on the brain region NO is either produced in the postsynapse and then diffuses into the presynaptic terminal (retrograde signalling) or *vice versa* (anterograde signalling). The eNOS in nearby vessels and nNOS in adjacent interneurons or other cells (indicated by the question marks) may represent alternative functionally relevant sources of NO. The cGKs (cGKI and cGKII) and members of two ion channel families (CNG and HCN channels) have been identified as downstream effectors of cGMP in synaptic plasticity. Retrograde NO signalling has been implicated in LTP in the hippocampus and amygdala. In the hippocampus, the cGKI is believed to localize to the presynapse and to phosphorylate various proteins (not shown) involved in cytoskeletal and vesicular dynamics. This promotes the clustering of vesicular proteins and proteins of the docking/fusion machinery (black triangles) leading to an increase in transmitter release and LTP. The cGKII has been suggested to act in hippocampal neurons postsynaptically to increase the expression of GluR1 homotetramers at the extrasynaptic surface. Anterograde NO signalling has been proposed for LTD in the cerebellum. Here, the cGKI is localized to the postsynapse, where it may enhance the internalisation of AMPA receptors indirectly through phosphorylation of G-substrate (not shown), which acts in its phosphorylated form as an inhibitor of protein phosphatases. This increases the level of phosphorylated AMPA receptors, which are removed from the postsynaptic membrane, thus, resulting in LTD of synaptic transmission. See text for further details.

Table 1 Synaptic plasticity in mouse models with a deletion of genes related to NO/cGMP signalling

Deleted gene	Mouse model	Phenotypes	References
nNOSα	Null mutation[a]	Reduced LTD in cerebellar parallel fibre synapses	(Lev-Ram et al. 1997b)
		Normal LTP in Schaffer collateral pathway	(O'Dell et al. 1994)
eNOS	Null mutation	Defective LTP in Schaffer collateral pathway	(Wilson et al. 1999)
		Defective LTP in mossy fibre pathway	(Doreulee et al. 2001)
		Defective LTP in cerebral cortex	(Haul et al. 1999)
eNOS + nNOSα	Null mutation[a]	Defective LTP in Schaffer collateral pathway and normal LTP in stratum oriens	(Son et al. 1996)
sGC α1	Null mutation	Reduced LTP in visual cortex	(Haghikia et al. 2007)
		Reduced LTP in hippocampus	(Koesling et al. 2007)
sGC α2	Null mutation	Reduced LTP in visual cortex	(Haghikia et al. 2007)
		Reduced LTP in hippocampus	(Koesling et al. 2007)
OCNG1	Null mutation	Reduced LTP in Schaffer collateral pathway	(Parent et al. 1998)
HCN1	Null mutation and forebrain-restricted mutation	Increased LTP in temporo-ammonic pathway and normal LTP in Schaffer collateral pathway	(Nolan et al. 2004)
cGKI	Null mutation	Normal LTP in Schaffer collateral pathway of young mice (4 weeks old)	(Kleppisch et al. 1999)
cGKII	Null mutation	Normal LTP in Schaffer collateral pathway of adult mice (12 weeks old)	(Kleppisch et al. 1999)
cGKI + cGKII	Null mutation	Normal LTP in Schaffer collateral pathway of young mice (4 weeks old)	(Kleppisch et al. 1999)
cGKI	Hippocampus-specific mutation	Reduced L-LTP in Schaffer collateral pathway of adult mice (12 weeks old)	(Kleppisch et al. 2003)
cGKI	Purkinje cell-specific mutation	Reduced LTD at the parallel fibre-Purkinje cell synapse	(Feil et al. 2003)
cGKI	Null mutation	Reduced LTP in the thalamic and cortical input of the lateral amygdala	(Paul et al. 2007)

[a] Note that the nNOS knockout mice were created by a deletion of exon 2 eliminating the splice variant nNOSα, which accounts for about 95% of the NOS activity in the whole brain. nNOS mutants retain significant NOS activity in the brain, likely reflecting the function of the remaining isoform, eNOS. However, this interpretation is complicated by the expression of two alternative splice variants lacking exon 2, nNOSβ and nNOSγ, in these mice (Eliasson et al. 1997).

support the conclusion that presynaptic NO signalling involves activation of its canonical pathway, i.e., stimulation of sGC followed by a rise of the cytosolic cGMP concentration and activation of cGK. LTP-inducing stimuli elicit an increase of cGMP in the hippocampus that is sensitive to NOS inhibitors and NO scavengers (Chetkovich et al. 1993). In addition, dibutyryl-cGMP can partially restore LTP in the presence of an NOS inhibitor (Haley et al. 1992). Importantly, several sGC inhibitors have been reported to reduce LTP (Arancio et al. 1995; Boulton et al. 1995; Zhuo et al. 1994). Evidence supporting a presynaptic localization of the cGMP signal came from studies of NMDA receptor-dependent LTP between pairs of cultured hippocampal neurons (Arancio et al. 1995). In this experimental system, injection of cGMP into the presynaptic neuron was sufficient to facilitate activity-dependent LTP.

As discussed in the sections above, cGKs represent a major class of cGMP receptors in the CNS. Initial evidence for a role of cGK in hippocampal LTP came from a study by Zhuo and coworkers (1994). These authors observed an enhancement of LTP following a weak tetanic stimulation in the presence of a cGK activator, while LTP was suppressed by cGK inhibitors. The function of individual cGKs, namely cGKI versus cGKII, remained unclear, since the pharmacological tools used in this study lacked selectivity. Two pieces of evidence hint at a predominant function of cGKI in presynaptic signalling. First, the expression level of cGKI in the hippocampus is much higher as compared to cGKII (de Vente et al. 2001; El-Husseini et al. 1995; Kleppisch et al. 1999). Second, cGKI has been detected in presynaptic terminals colocalized with the vesicle protein synaptophysin (Arancio et al. 2001). The concept of presynaptic cGMP signalling via cGKI was reinforced by studies of NMDA receptor-dependent LTP between pairs of cultured hippocampal neurons (Arancio et al. 2001): injection of purified cGKIα protein into the presynaptic neuron facilitated LTP induced by a weak tetanus, while a cGK inhibitor injected into the presynaptic neuron blocked LTP. Fitting well with the model of retrograde NO signalling, postsynaptic infusion of these compounds had no effect.

At odds with the findings described above, juvenile mice (4 weeks old) lacking cGKI, cGKII or both (double knockout mice) are capable of normal LTP in the Schaffer collateral pathway (Kleppisch et al. 1999) (Table 1). Yet, these observations in cGK null mutants bear the uncertainty that potential defects in LTP may be masked due to functional compensation or phenotypes related to other organ systems. Most conventional cGKI knockout mice die until 6–8 weeks of age due to multiple smooth muscle-related defects including gastrointestinal dysfunction (Pfeifer et al. 1998; Weber et al. 2007). Their premature death prevented the analysis of hippocampal functions and behaviour in adult cGKI null mice. The generation of hippocampus-specific cGKI knockout ($cGKI^{hko}$) mice eliminated these problems (Kleppisch et al. 2003). However, the adult $cGKI^{hko}$ mice also showed normal hippocampal LTP in response to a single theta burst stimulation (Kleppisch et al. 2003). The original description of hippocampal LTP (Bliss and Lomo 1973) marked the fact that repeated delivery of conditioning stimuli resulted in synaptic potentiation of greater magnitude and persistence. Later it became clear that multiple strong tetanic stimulation of Schaffer collaterals results in a protein synthesis-

dependent late phase LTP (L-LTP) mechanistically distinct from the more decremental early phase of LTP (Barco et al. 2002; Frey et al. 1988). Interestingly, cGMP has been implicated in L-LTP (Lu and Hawkins 2002; Lu et al. 1999). Fitting well with the view that cGKI acts as a downstream target of cGMP in L-LTP, LTP following multiple episodes of strong theta burst stimulation was decreased in adult $cGKI^{hko}$ mice (12 weeks old) (Kleppisch et al. 2003). Moreover, the protein synthesis inhibitor anisomycin had no effect on the residual LTP in $cGKI^{hko}$ mice, while it decreased LTP in control mice to the level observed in the mutants. Recently, cGKII has been reported to support hippocampal LTP by a postsynaptic mechanism (Serulle et al. 2007) fundamentally different from that of cGKI. The molecular mechanisms downstream of cGKI and cGKII are discussed below in Sect. 4.

It is unlikely that all effects of cGMP are mediated via cGKs. In addition, cytosolic cGMP can activate members of two ion channel families, cyclic nucleotide-gated (CNG) and hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels (Hofmann et al. 2005), leading to membrane depolarization and excitation. CNG channels are critically involved in visual and olfactory signal transduction (Biel et al. 1999; Hofmann et al. 2004). They are also found in brain regions important for learning and memory and have been implicated in the regulation of synaptic plasticity (Barnstable et al. 2004; Bradley et al. 1997; Strijbos et al. 1999; Zufall et al. 1997). For instance, Ca^{2+}-permeable olfactory CNG channels (OCNG1), which are activated by cAMP and cGMP, are expressed in the hippocampus and support LTP in the Schaffer collateral pathway (Parent et al. 1998). Similar to CNG channels, members of the HCN channel family may serve as effective presynaptic transducers of cGMP signals as described recently for rat optic nerve axons (Garthwaite et al. 2006). According to an alternative view, HCN1 channels modulate the integration of excitatory synaptic inputs to distal dendrites of postsynaptic CA1 neurons and, thus, constrain synaptic plasticity and learning (Nolan et al. 2004; Tsay et al. 2007).

In addition to LTP at Schaffer collateral/CA1 synapses, cGMP signalling may be involved in a form of hippocampal long-term *depression* that requires mobilization of Ca^{2+} from ryanodine-sensitive stores in presynaptic terminals (Reyes-Harde et al. 1999a, b; Reyes and Stanton 1996). In this case, cGMP has been suggested to activate ADP-ribosyl cyclase which generates cyclic ADP ribose, an endogenous activator of ryanodine receptors. Induction of this form of LTD also requires a cGK-mediated step (Reyes-Harde et al. 1999b), perhaps the phosphorylation of ADP-ribosyl cyclase. How *increased* Ca^{2+} release from ryanodine-sensitive presynaptic stores ultimately causes a *decrease* in transmitter release remains unknown.

The $cGKI^{hko}$ mice represent an excellent model to answer the question whether there is a functional link between cGMP-induced synaptic plasticity and hippocampus-dependent behaviour. Surprisingly, the impaired hippocampal L-LTP in adult $cGKI^{hko}$ mice had no impact on their performance in two tests of hippocampus-dependent learning, contextual fear conditioning and spatial learning in a discriminatory water maze (Kleppisch et al. 2003). These findings strongly suggest that the cGMP/cGKI pathway modulates hippocampal LTP but is not critical to distinct forms of hippocampus-dependent learning. However, these data do not

exclude the possibility that cGMP signalling plays important roles in other forms of learning and memory. Indeed, both sGC and cGKI mouse mutants show a number of behavioural phenotypes that are presumably related to brain regions other than the hippocampus (see Sect. 3.4). Interestingly, mice with a global deficiency of endothelial NO synthase (eNOS) have been reported to show improved learning in the hidden-platform water maze task (Frisch et al. 2000) most likely reflecting alterations in stress coping rather than in spatial learning. This is supported by the observation that eNOS-deficient mice showed normal spatial learning in an alternative, less stressful radial maze task (Dere et al. 2001).

3.2 Amygdala

Learning and remembering of fearful events depends on the integrity of the amygdala, specifically the lateral nucleus of the amygdala (LA), and can be assessed by fear conditioning (for review see Maren and Quirk 2004). In this experimental model, animals learn to associate an initially neutral conditioned stimulus (CS, routinely a tone) with danger represented by a simultaneously applied aversive unconditioned stimulus (US, routinely a foot-shock that induces freezing behaviour). The fact that somatosensory (US) and auditory (CS) inputs convergence on individual principal neurons in the LA stimulated the cellular hypothesis of fear learning (Blair et al. 2001; Sigurdsson et al. 2007): behavioural changes are due to a persistent increase of synaptic transmission in auditory inputs induced by simultaneous strong activity of nearby somatosensory inputs. This hypothesis implicates that associative fear memory and LTP in the LA share common molecular mechanisms (Sigurdsson et al. 2007). The role of cGMP signalling in these processes will be summarized below.

As for the hippocampus, cGMP was assigned a role in synaptic plasticity and fear learning in the amygdala in association with NO. Monitoring of NO oxidation products *in vivo* indicated that NO levels in the amygdala rise following fear conditioning and correlate with freezing behaviour (Sato et al. 2006). There is further evidence that effectors of NO relevant to synaptic plasticity and auditory-cued fear memory, such as sGC and cGK, are located presynaptically in the LA (Chien et al. 2003, 2005; Huang and Kandel 1998; McKernan and Shinnick-Gallagher 1997; Schafe et al. 2005; Tsvetkov et al. 2002; Watanabe et al. 1995). YC-1, a substance facilitating activation of sGC by NO (Friebe et al. 1998), markedly enhanced LTP in the hippocampus and amygdala, and rats treated with YC-1 exhibited a superior performance in an amygdala-related aversive avoidance test compared with controls. Inhibitors of either NOS (L-NAME) or cGK (KT5823 and Rp-8-Br-PET-cGMPS) applied to the cerebrospinal fluid were able to suppress this effect of YC-1 (Chien et al. 2005). *In situ* hybridization and immunohistochemical data showed that both cGKI and cGKII are expressed in the amygdala (de Vente et al. 2001; El-Husseini et al. 1995, 1999; Feil et al. 2005b; Geiselhoringer et al. 2004; Oster et al. 2003; Werner et al. 2004). A specific function of the cGKI in the amygdala was pointed out

recently (Paul et al. 2007): cGKI null mutants showed reduced LTP in the cortical and thalamic input to the LA, and this electrophysiological phenotype correlated with an impairment of long-term fear memory. For the behavioural studies, the authors used so-called cGKI smooth muscle rescue mice (Weber et al. 2007) that lack cGKI in the nervous system and do not show the severe gastrointestinal defects of null mutants. The behavioural phenotype reflects a weakened association of the tone with the shock in the absence of cGKI. In contrast, the cGKI mutant mice showed normal levels of freezing in the conditioning context (Paul et al. 2007). These data indicate that cGKI has a specific function in auditory-cued fear memory, but not contextual fear memory thought to be dependent on the hippocampus. A contribution of cGKI-deficiency in other brain regions to the behavioural phenotype of the cGKI smooth muscle rescue mice cannot be ruled out. However, the following findings support the view that the enzyme's function in auditory-cued fear memory is specifically related to the LA. First, the level of cGKI expression in the LA is among the highest in the brain, while its expression in the thalamus, another important part of the fear conditioning pathway, appears to be extremely low (Paul et al. 2007). Second, the $cGKI^{hko}$ mice which have a deletion of the cGKI gene in principal neurons of the cortex and the hippocampus show normal auditory-cued and contextual fear memory (Kleppisch et al. 2003). Further support for a role of cGMP in the amygdala comes from the recent finding that distinct changes in social behaviour in monkeys correlate with an up regulation of the sGC in the amygdala (Sabatini et al. 2007).

3.3 Cerebellum

Purkinje cells (PCs) provide the sole inhibitory output of the cerebellar cortex to vestibular and cerebellar nuclei serving important functions in motor coordination and reflexive behaviours, and learned adaptations of these behaviours. The large dendritic tree of an individual PC in the molecular layer of the cerebellar cortex normally receives excitatory inputs from a single climbing fibre (CF) and numerous parallel fibres (PFs) originating from granule cells within the granular layer (for review see Ito 2001). Motor learning is thought to be based on LTD of the glutamatergic synaptic transmission from PFs onto PCs (Carey and Lisberger 2002; Mauk et al. 1998; Raymond et al. 1996). Similar to LTP in the hippocampus, cerebellar LTD can be induced experimentally by specific activity patterns. It is also long known that PCs show robust expression of cGKI (Hofmann and Sold 1972; Lohmann et al. 1981). Therefore, it is not surprising that the analysis of cGMP signalling in the cerebellum was focussed on PCs and cGKI.

LTD in PF–PC synapses is evoked when PFs are repeatedly activated in conjunction with the CF converging onto the same PC. The coincidence of a rise in postsynaptic cGMP and Ca^{2+} is sufficient to induce LTD (Lev-Ram et al. 1995, 1997a). The production of cGMP is initiated by NMDA receptor-dependent generation of NO, presumably in interneurons (Shin and Linden 2005), and subsequent

stimulation of sGC in PCs. LTD can be blocked by inhibitors of NOS or sGC, while application of membrane-permeable cGMP analogues restores LTD (Boxall and Garthwaite 1996; Hartell 1994; Lev-Ram et al. 1997a). Moreover, cerebellar LTD is abolished in nNOS mutant mice (Lev-Ram et al. 1997b). In contrast to the situation in the hippocampus and amygdala, a postsynaptic source for NO triggering cerebellar LTD is highly unlikely, since neither NMDA receptors nor nNOS were detected in postsynaptic PCs. Instead, it is thought that NO acts as an anterograde messenger that is produced in presynaptic PF terminals or interneurons (Shin and Linden 2005) and then diffuses into the postsynaptic PC to activate a cGMP-dependent signalling pathway (Fig. 1) (Ito 2001). The cGMP receptor cGKI is thought to serve a key function in cerebellar LTD. Its role was first suggested based on findings that inhibitors of the enzyme (e.g., KT5823) impaired LTD in cerebellar slices (Hartell 1994; Lev-Ram et al. 1997a). In vivo proof for this model was provided using a genetic approach, namely mice with a PC-specific disruption of the cGKI gene (cGKI^{pko} mice) (Feil et al. 2003). These mutants showed a normal morphology of the cerebellar cortex and a normal Ca^{2+} signal evoked by CF activation indicating that PCs lacking cGKI show a normal CF innervation and no generalized structural or physiological abnormalities. However, inactivation of the cGKI gene in PCs virtually abolished LTD in PF-PC synapses in acute slice preparations (Feil et al. 2003) (Table 1). Thus, cGKI appears to be the central cGMP effector in cerebellar LTD.

LTD of the PF-PC synapse in the cerebellum has been suggested to underlie certain forms of motor learning (Ito 2001). What is the role of cGMP signalling for motor learning? General motor performance, assessed by footprint patterns and the runway and rotarod tests, was not affected by the lack of cGKI in PCs (Feil et al. 2003). These results replicate findings in nNOS-deficient mice which exhibit a defect in cerebellar LTD but not in basal motor learning (Lev-Ram et al. 1997b). PCs possess a unique feature: they represent the ultimate convergence point for inputs from the vestibular organ (via mossy fibre and PF pathway) and the retinas (via CF pathway). Therefore, it has been proposed that cerebellar LTD is involved in the adaptation of the vestibulo-ocular reflex (VOR), which keeps images stable on the retina during head movements. In line with this view, nNOS knockout mice and cGKI^{pko} mouse mutants exhibited marked deficits in the adaptation of compensatory eye movements (Katoh et al. 2000) and adaptation of the VOR (Feil et al. 2003), respectively. The phenotypes of cGKI^{pko} mice demonstrate that cGKI in cerebellar PCs has a specific function in signalling pathways related to synaptic plasticity and adaptation of the VOR, while it is dispensable for general motor coordination. In line with the phenotype of cGKI^{pko} mice, the pharmacological prevention of cerebellar LTD in rodents did not affect motor learning in several standard tests (Welsh et al. 2005). Together, these findings support the notion that cerebellar LTD is critically involved in specific forms of motor learning (e.g., the adaptation of the VOR), but is not essential for basal motor performance. Synaptic plasticity in other brain regions, such as the striatum, may also support motor learning (Dang et al. 2006). It is important to note that the cGKI^{pko} mice lacked the gene of interest selectively in cerebellar PCs and showed a normal CF innervation (Feil et al. 2003). Thus, it can be excluded that the defect in adaptation of the VOR was simply due to aberrant

multiple CF innervation of PCs as described for motor phenotypes in other mouse mutants (Ito 2001). Potential postsynaptic signalling mechanisms downstream of cGMP/cGKI thought to support LTD and cerebellum-dependent learning will be discussed in detail in Sect. 4.2.

3.4 Other Brain Regions and Complex Behaviours

The application of GMP-elevating agents or cGMP analogues, as well as the genetic deletion of cGMP generators and effectors, results in marked behavioural alterations in rodents. Since it is difficult to assign these phenotypes to cGMP signalling in specific areas of the CNS, the behavioural studies are summarized in this section under "other brain regions and complex behaviours". Note, however, that this does not exclude a role of the hippocampus, cerebellum or amygdala in the respective behaviours.

Recently, the initial characterization of the neuronal phenotypes of sGC knockout mice was published (Haghikia et al. 2007) (Table 1). The NO-dependent LTP of synaptic transmission in the visual cortex was lost in both $\alpha_1{}^{-/-}$ as well as $\alpha_2{}^{-/-}$ mice indicating that each sGC isoform plays a distinct role in LTP and is localized to a specific functional compartment, perhaps one isoform in the presynapse and the other in the postsynapse or in interneurons (Szabadits et al. 2007). This result contrasts with findings in the vascular system, where the α_1- and α_2-containing sGC isoforms can substitute for each other (Mergia et al. 2006). In line with the phenotype of hippocampus-specific cGKI knockout mice (see Sect. 3.1), both sGC isoform mutants had reduced LTP in the hippocampus, while spatial learning analysed in a water maze test was not affected (Koesling et al. 2007). However, other behavioural alterations are clearly present in sGC knockout mice, e.g., in active avoidance conditioning and fear conditioning (Koesling et al. 2007), as well as in cGKI mutant mice. Preliminary data from behavioural phenotyping of mouse mutants lacking cGKI in the nervous system suggest that this protein kinase is critically involved in the formation of fear memory (Paul et al. 2007), in social and object recognition, and in the regulation of sleep-wake activity (Feil et al. 2007). Taken together, it can be concluded that the sGC/cGMP/cGKI pathway has little relevance for spatial learning, but is involved in the modulation of other cognitive functions in mammals.

Pharmacological and genetic studies suggest important roles for cGMP-related PDEs in cognition, psychosis, depression, and neurodegeneration (Menniti et al. 2006). For instance, inhibition of PDE2 with Bay 60-7550 (Boess et al. 2004) as well as inhibition of PDE5 with sildenafil or vardenafil (Prickaerts et al. 2002) improved the performance of rats in social and object recognition memory tasks. However, PDE5 inhibition had no effect on spatial learning (Prickaerts et al. 2004). The results obtained with PDE inhibitors are in good agreement with the phenotypes of sGC and cGKI knockout mice, but the neuronal substrates and mechanisms for the procognitive effects of PDE inhibitors remain to be determined. Fitting well with a role in learning and memory, the PDE2 is highly expressed in the limbic system

(Repaske et al. 1993). Since PDE2 is a dual-substrate PDE that hydrolyzes both cGMP and cAMP, it cannot be excluded that at least some effects of PDE2 inhibition are mediated through cAMP- rather than cGMP-regulated signalling cascades. Interestingly, the expression of PDE5 in the brain appears to be restricted mainly to cerebellar PCs (Kotera et al. 1997, 2000) supporting a role of the cerebellum in cognition (Rapoport et al. 2000). It is tempting to speculate that cGMP-elevating drugs may be used to treat cognitive impairment associated with neurodegenerative disorders or hepatic encephalopathy. Indeed, chronic administration of sildenafil was reported to restore learning ability in rats with hyperammonemia (Erceg et al. 2005).

Sildenafil has also been associated with aggressive behaviour in mice (Hotchkiss et al. 2005) pointing to CNS adverse effects of its clinical use in humans (Milman and Arnold 2002). Moreover, studies with sildenafil and cGMP analogues thought to inhibit cGK activity have implicated cGMP and cGKs in the modulation of anxiety (Li et al. 2005; Volke et al. 2003). Interestingly, cGKII knockout mice showed increased anxiety-like behaviour, reduced sensitivity to the hypnotic effect of ethanol, and augmented voluntary consumption of ethanol (Werner et al. 2004). These phenotypes suggest the existence of an anxiolytic cGMP/cGKII pathway and support the notion that anxiety-like behaviour correlates with ethanol consumption (Henniger et al. 2002). Cyclic GMP has also been linked to the regulation of dopaminergic neurons in regions involved in addictive behaviour like the striatum and the ventral tegmental area. Activation of the cGMP pathway reduced cocaine-induced gene expression in dopaminergic structures as well as cocaine-related effects on behaviour in rats (Jouvert et al. 2004). A recent electrophysiological study identified a novel form of LTP in the rat ventral tegmental area that is initiated by NO/cGMP and blocked by morphine (Nugent et al. 2007). Thus, interruption of cGMP signalling may contribute to neuroadaptations to opioid drugs and addiction. A potential role of cGMP in drug abuse is further supported by a genome wide study that found an association of the human cGKI gene with nicotine dependence (Uhl et al. 2007). Studies in rodents and humans indicate that components of the cGMP signalling system may also play a role in the pathophysiology of psychiatric diseases, for instance, PDE9 in bipolar affective disorder (Straub et al. 1994), PDE11 in major depression (Wong et al. 2006), PDE10 in social interaction and schizophrenia (Sano et al. 2008; Schmidt et al. 2008), and cGKI in seasonal affective disorder (Sokolowski 2007) (see also the chapter by Kleppisch, this volume).

It is interesting to note that cGMP and, particularly, cGKs, are also modifiers of complex behaviours in invertebrates (see also the chapter by Sokolowski et al., this volume). In insects and nematodes, cGKs have important functions in foraging and sensory adaptation (Ben-Shahar et al. 2002; Fujiwara et al. 2002; L'Etoile et al. 2002; Osborne et al. 1997). In accordance with the phenotypes of cGK mouse mutants, the *Drosophila* cGK is also involved in certain forms of learning and memory (Mery et al. 2007) and the *C. elegans* cGK has been reported to regulate sleep-like behaviour (Raizen et al. 2008). Although it remains to be established how many parallels can be drawn between particular cGK functions in worms, flies, mice and man, it is clear that the cGKs are important modulators of behavioural plasticity in both invertebrates and mammals.

4 Molecular Mechanisms Underlying cGMP-Induced Changes of Synaptic Plasticity

Principally, changes of glutamatergic synaptic transmission observed in LTP and LTD and associated learning and memory can be based on pre- and/or postsynaptic mechanisms (for review see Bear and Malenka 1994; Larkman and Jack 1995; Lisman and Raghavachari 2006). While presynaptic mechanisms are thought to converge on the modulation of glutamate release, postsynaptic mechanisms should ultimately alter the response to a given quantum of transmitter released. The following sections discuss pre- and postsynaptic mechanisms of cGMP-dependent synaptic plasticity (Fig. 1) including the proteins that are potentially phosphorylated by cGKs in various brain regions. For a comprehensive review of cGK substrates, see the chapter by Lincoln et al., this volume.

4.1 Regulation of Presynaptic Transmitter Release

A presynaptic locus has been implied in the expression of LTP in the hippocampus and amygdala (Apergis-Schoute et al. 2005; Arancio et al. 1995, 1996; Tsvetkov et al. 2002). There is considerable evidence that cGMP signalling supports an increase in presynaptic transmitter release (for review see Feil and Kleppisch 2008). What are the underlying mechanisms? Modulation of transmitter release may be based on changes in the mode of vesicle fusion, the number of vesicles released in response to afferent stimulation, and vesicle recycling. Work by Micheva and colleagues (2001, 2003) showed that retrograde signalling via NO promotes synaptic vesicle recycling in CNS neurons in an activity-dependent manner. The authors monitored exo- and endocytosis in cultured hippocampal neurons using a pH-sensitive GFP reporter protein and the fluorescent dye FM4-64. Based on fluorescence imaging and pharmacological analysis, they proposed that NMDA receptor-dependent postsynaptic production of NO accelerates vesicle endocytosis by increasing the level of presynaptic phosphatidylinositol 4,5-bisphosphate (PIP_2) via a cGMP-dependent mechanism. PIP_2 is a phospholipid that has been extensively implicated in vesicle cycling (Martin 2001; Osborne et al. 2001), but how NO/cGMP leads to an increase in PIP_2 at the synapse is not clear. Studies in non-neuronal cells suggest that the NO/cGMP/cGK pathway can inhibit the PIP_2-hydrolyzing phospholipase C (Clementi et al. 1995; Xia et al. 2001).

cGK targets that are likely to link cGMP signalling to vesicle function include the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Ninan and Arancio 2004), the vasodilator-stimulated phosphoprotein (VASP) (Wang et al. 2005), the small GTPase RhoA, and α-synuclein (Liu et al. 2004). The CaMKII is expressed in hippocampal neurons and plays an important role in synaptic plasticity. While previous interest was focussed on its postsynaptic function (for review see Lisman et al. 2002), a recent study elucidated the presynaptic function of CaMKII in cGMP-regulated synaptic plasticity in cultured hippocampal neurons (Ninan and Arancio

2004): presynaptic injection of a membrane-impermeable CaMKII inhibitor peptide blocked LTP of synaptic transmission induced by combining a weak tetanus with brief superfusion of a NO donor or cGK activator. Moreover, the CaMKII inhibitor peptide suppressed the increase in the number of active presynaptic boutons occurring normally under the same experimental conditions. However, it is important to note that the major mechanism activating CaMKII is presynaptic Ca^{2+} entry, which by itself is sufficient to induce LTP independent of cGMP. Even more confusing, data from a study in mutant mice lacking the CaMKII α subunit selectively in presynaptic CA3 neurons of the Schaffer collateral pathway implicate an *inhibitory* rather than *stimulatory* function of the enzyme in activity-dependent transmitter release (Hinds et al. 2003). Without doubt, further studies are needed to resolve the relationship between cGMP signalling, CaMKII activity and presynaptic transmitter release.

Actin filament reorganization in dendritic spines has been suggested to represent an important mechanism for the induction of LTP (Fukazawa et al. 2003; Lisman 2003; Lisman and Raghavachari 2006; Wang et al. 2005). Remarkably, activity-dependent cytoskeletal changes also occur at the presynaptic site and promote a rapid increase in clusters of the vesicular proteins synaptophysin and synapsin I (Antonova et al. 2001; Wang et al. 2005). Two well-known regulators of actin dynamics, VASP and RhoA, are putative downstream targets of cGMP/cGK signalling. Studies with cultured hippocampal neurons (Arancio et al. 2001; Wang et al. 2005) showed that VASP is an endogenous cGK substrate in hippocampal neurons and that cGK-dependent VASP phosphorylation is a critical step in the induction of LTP (Wang et al. 2005). Components of the cGMP signalling cascade, such as sGC, cGKI and VASP, are expressed in presynaptic terminals, and synaptic potentiation is associated with VASP phosphorylation at Ser-239, which serves as a biomarker for cGK activity (Arancio et al. 2001). However, the assumption that VASP phosphorylation by cGK is critical for activity-dependent changes in transmitter release is challenged by the fact that mice lacking VASP or related proteins show no phenotypes of synaptic plasticity (Hauser et al. 1999; Reinhard et al. 2001). A functional role of RhoA was supported by the finding that the general inhibitor of Rho GTPases, clostridium difficile toxin B, reduced glutamate-induced synaptic potentiation and the associated increase in synaptophysin puncta in cultured hippocampal neurons (Wang et al. 2005), while toxin B had no effect on basal synaptic transmission. The cGK activator 8-pCPT-cGMP was able to restore synaptic potentiation in the presence of a NOS inhibitor and toxin B. The latter finding suggests that Rho GTPases act *upstream* rather than downstream of cGMP during glutamate-induced synaptic potentiation.

It has been suggested that a NO-dependent mechanism strengthens the stimulatory function of α-synuclein, a protein linked to neurodegeneration, on transmitter release (Liu et al. 2004). Long-lasting potentiation of synaptic transmission between cultured hippocampal neurons was accompanied by an increase in the number of presynaptic clusters of α-synuclein. Moreover, suppressing α-synuclein expression (e.g., by antisense or knockout techniques) blocked both synaptic potentiation and the increase in the presynaptic number of functional boutons normally associated with it. Remarkably, presynaptic injection of α-synuclein caused rapid and

long-lasting enhancement of synaptic transmission and rescued potentiation in cultures from mice carrying an α-synuclein null mutation (Liu et al. 2004). Exogenous NO increased the number of α-synuclein clusters and this effect was blocked by NOS inhibitors (Liu et al. 2004). However, the view that α-synuclein indeed links NO/cGMP signalling to increased transmitter release needs to be substantiated by further experiments. The data discussed above indicate that cGMP signalling may promote coordinated changes in the distribution of proteins in the presynaptic terminal functionally related to transmitter release. These changes may increase the efficacy of synaptic transmission by supporting the alignment of presynaptic release sites with postsynaptic receptors.

Transmitter release can also be regulated by an autoinhibitory feedback via presynaptic G protein-coupled receptors (GPCRs) (cf. Starke 1981). The GTPase activity of the G protein α-subunit is boosted by RGS (regulator of G protein signalling) proteins resulting in accelerated termination of GPCR-mediated responses. Currently, there are more than 30 RGS proteins known (for review see Hollinger and Hepler 2002), and members of subfamilies 2, 3 and 4 are cGK substrates (Pedram et al. 2000; Tang et al. 2003). In the vasculature, NO/cGMP-dependent effects have been linked to phosphorylation of RGS2 by cGKI (Sun et al. 2005; Tang et al. 2003). Morphological and electrophysiological analyses emphasize that RGS2 is also an important regulator of transmitter release and synaptic plasticity in hippocampal neurons (Han et al. 2006; Ingi et al. 1998; Oliveira-Dos-Santos et al. 2000) and in dopaminergic neurons of the ventral tegmental area (Labouebe et al. 2007). Based on these findings it is tempting to speculate about a modulation of presynaptic transmitter release through phosphorylation of RGS2 by cGKI.

Another cGK substrate that plays a role in presynaptic transmitter homeostasis is the human serotonin transporter (hSERT) (Zhu et al. 2004) which removes 5-hydroxytryptamine from the synaptic cleft and is a prominent target for therapeutic intervention in mood, anxiety, and obsessive-compulsive disorders. Interestingly, hSERT variants which may be associated with neuropsychiatric disorders display altered sensitivity to regulation by cGK (Prasad et al. 2005; Zhang et al. 2007).

The presynaptic cGMP signalling mechanisms for modulating transmitter release discussed so far are thought to require activation of cGK (Fig. 1). Evidence from experiments in cones of the salamander retina suggests that exocytosis can also be triggered by a Ca^{2+} influx through CNG channels (Rieke and Schwartz 1994; Savchenko et al. 1997). CNG channels are expressed in the hippocampus and other regions of the mammalian brain. They may, therefore, serve as universal transducers linking activity-dependent generation of cGMP in the presynaptic terminal to enhanced transmitter release (Bradley et al. 1997; Kingston et al. 1996).

4.2 Regulation of Postsynaptic Function

Fast excitatory transmission in the CNS is mediated through postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. It has emerged

that their activity-regulated trafficking represents a fundamental mechanism for the modification of synaptic strength and learning (for review see Bredt and Nicoll 2003; Derkach et al. 2007; Malinow and Malenka 2002; Rumpel et al. 2005). For example, rapid insertion of AMPA receptors (AMPARs) into the synaptic region and/or into the extrasynaptic membrane with subsequent redistribution via diffusion to the synaptic region plays an import role for the expression of LTP. Conversely, rapid internalisation of AMPARs from these sites decreases synaptic strength and has been reported to contribute to the expression of LTD in the cerebellum (Man et al. 2000; Wang and Linden 2000). AMPARs are homo- or heterotetramers of the subunits GluR1–4 (Dingledine et al. 1999; Hollmann and Heinemann 1994; Wisden and Seeburg 1993). Besides regulated trafficking, LTP may also involve activity-regulated dynamic changes in the subunit composition of AMPARs (Plant et al. 2006). For instance, the fraction of GluR1 homotetramers strongly increases shortly after the induction of hippocampal LTP. Unlike their heteromeric counterparts, GluR1 homotetramers are Ca^{2+}-permeable and, thus, provide a pathway for a Ca^{2+} influx thought to be important for the maintenance of LTP. A recent study with cultured hippocampal neurons suggested that cGMP regulates AMPAR trafficking and subunit composition via cGKII (Serulle et al. 2007). According to these authors, cGKII and GluR1 colocalize and interact in a cGMP-stimulated manner resulting in phosphorylation of GluR1 and, ultimately, an increase of AMPAR surface expression at extrasynaptic sites. Both the increase in AMPARs and the associated increase in synaptic strength were blocked by a cGKII inhibitor peptide as well as by other cGK inhibitors with limited selectivity for the cGKII isoform. Taken together, these findings support the following scheme for postsynaptic cGMP signalling: cGMP causes a conformational change of the cGKII that promotes binding to and subsequent phosphorylation of the AMPAR subunit GluR1 which, in turn, facilitates its insertion into the membrane. Such GluR1 targeted interaction is expected to increase the fraction of GluR1 homotetramers at the postsynaptic site and, thus, synaptic strength (Fig. 1).

In contrast to hippocampal LTP, a cGMP-dependent mechanism for rapid *internalisation* of postsynaptic AMPARs is thought to underlie cerebellar LTD. As discussed above, the cGKI in PCs has a critical function in cerebellar LTD (see Sect. 3.3), but what are the downstream effectors of the cerebellar cGMP/cGKI signalling cascade? Induction of LTD is thought to involve an increase in clathrin-mediated endocytosis of AMPARs, which is facilitated by AMPAR phosphorylation, for instance, by protein kinase C (Chung et al. 2003; Wang and Linden 2000). Alternatively, decreased phosphatase activity should also result in an increased level of phosphorylated AMPARs and their removal from the postsynaptic membrane. Intriguingly, cGMP/cGKI signalling inhibits the phosphatase limb via phosphorylation of G-substrate, a well-characterized cGK substrate expressed in PCs (Ito 2002). Phosphorylated G-substrate suppresses the activity of protein phosphatase 1/2A (Hall et al. 1999), which has been linked to LTD induction (Ajima and Ito 1995). Thus, the following model of cerebellar LTD (Fig. 1) and motor learning can be proposed: NO/cGMP-dependent activation of cGKI results in phosphorylation of G-substrate in PCs and subsequent inhibition of protein phosphatases, which in turn results in an increased level of AMPAR phosphorylation and endocytosis and, thus,

induction of LTD. Future studies assessing the effects of phosphatase inhibitors on LTD in cGKI^{pko} mice, will help to validate this model.

Another interesting cGK substrate is the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) (Nishi et al. 2005; Tsou et al. 1993). The phosphorylated form of DARRP-32, similar to G-substrate in cerebellar PCs, is a potent protein phosphatase inhibitor. DARPP-32 is localized to neurons containing dopamine receptors, particularly medium spiny neurons in the neostriatum, and is thought to play a pivotal role in the integration of dopaminergic and glutaminergic signalling, cognitive function and possibly the pathogenesis of schizophrenia (Meyer-Lindenberg et al. 2007; Svenningsson et al. 2004).

4.3 Regulation of Gene Expression

There is considerable evidence that cGMP modulates gene expression in non-neuronal and neuronal cells (Pilz and Casteel 2003). Ca^{2+}-dependent pathways that control transcription via cAMP response element binding protein (CREB) and nuclear factor of activated T-cells (NFAT) function as important molecular switches in the formation of L-LTP and long-term memory in various regions of the CNS (Barco et al. 2002; Chen et al. 2003; Josselyn et al. 2001, 2004; Josselyn and Nguyen 2005). It has been reported that cGMP can activate the transcription factor CREB in the hippocampus and perhaps also in the amygdala (Chien et al. 2003; Lu and Hawkins 2002; Lu et al. 1999). These effects may be mediated via cGKs (Lu and Hawkins 2002), but the complete signalling cascade for cGMP activation of CREB-mediated gene transcription remains to be elucidated. A recent report suggested the interesting possibility that the cognitive decline in Alzheimer's disease might, at least in part, be related to inhibition of hippocampal cGMP/CREB signalling by amyloid-β peptide (Puzzo et al. 2005). The cGMP/cGK pathway has been shown to modulate calcineurin-regulated NFAT in non-neuronal tissues (Fiedler et al. 2002; Gonzalez Bosc et al. 2004). For instance, in vascular smooth muscle cGK promotes the nuclear localization of NFATc3 indirectly by inhibiting c-Jun terminal kinase 2, thereby, reducing NFAT phosphorylation and the associated export of the phospho-protein from the nucleus (Gonzalez Bosc et al. 2004). Members of the NFAT family are expressed in the CNS and have been reported to support synaptic plasticity (Graef et al. 1999; Ho et al. 1994). It is, therefore, feasible that a cGMP/cGK-dependent mechanism similar to that described for smooth muscle also contributes to the formation of L-LTP and long-term memory in the CNS. Noteworthy, such an indirect mechanism for NFATc3 nuclear accumulation would require a rise of both cytosolic Ca^{2+} and cGMP.

Regulation of gene expression by cGKs has also been shown to participate in other forms of neuronal plasticity such as long-term sensitization of nociception (Lewin and Walters 1999). Remarkably, the development of chronic hyperalgesic states (e.g., inflammation-induced hyperalgesia) partially relies on activation of NFAT-dependent transcription of pro-nociceptive genes by neurotrophins

(e.g., BDNF) in primary afferent and spinal neurons (Groth et al. 2007; Groth and Mermelstein 2003). Likewise, neutrophins can act through the transcription factor CREB (Finkbeiner et al. 1997). Collectively, these findings hint at a role for cGMP/cGK in activating NFAT and CREB with possible functional relevance for hyperalgesia.

What genes are expressed in a CREB- or NFAT-dependent manner? Actually, the list of these genes is long, including transcription factors, membrane channels and transporters, cytokines, structural proteins and proteins involved in neurotransmission, metabolism, and axonal growth (Canellada et al. 2006; Graef et al. 2003; for review see Lonze and Ginty 2002). However, it remains unclear which of these genes are indeed regulated by cGMP signalling. As genes regulated by CREB and NFAT can be associated with presynaptic as well as postsynaptic functions, there is also no universal rule regarding the locus of cGMP/cGKI-dependent regulation of gene transcription.

5 Conclusion

A large body of evidence demonstrates a modulatory function of cGMP in synaptic plasticity and behavioural responses. Typically, cGMP signalling in the brain is initiated by NO via sGC and transmitted through cGKs, CNG and HCN channels, and PDEs. The cellular location of the respective pathway components relevant for synaptic plasticity and learning may be both presynaptic and postsynaptic. cGMP has been suggested to promote presynaptic transmitter release through regulation of Ca^{2+} signalling and cytoskeletal dynamics. Postsynaptic effects of cGMP are presumably related to altered trafficking and subunit composition of AMPARs. In hippocampal pyramidal cells, cGKII-mediated phosphorylation of GluR1 increases its surface expression. In cerebellar PCs, cGKI may increase the phosphorylation and endocytotic removal of AMPARs from the postsynaptic membrane indirectly via the inhibition of protein phosphatases. Long-term effects of cGMP on synaptic activity and behaviour may also be mediated via the regulation of gene expression in various brain regions. Dysfunction of cGMP signal transduction appears to be involved in the pathogenesis of neurodegenerative and psychiatric diseases with cognitive impairments, such as Morbus Alzheimer and schizophrenia. Hence, components of cGMP signalling are of great interest as targets for the development of new drugs to the treat these disorders. Encouraging preclinical findings in this direction have been reported for both PDE inhibitors and sGC stimulators (Chien et al. 2005; Feil and Kemp-Harper 2006; Kemp-Harper and Feil 2008; Menniti et al. 2006)

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