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Vsevolod V. Gurevich *Editor*

Arrestins - Pharmacology and Therapeutic Potential

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Arrestins - Pharmacology and Therapeutic Potential

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Preface

The first arrestin was discovered in the visual system as a key player in the shutoff of prototypical G protein-coupled receptor (GPCR) rhodopsin. Cloning and functional characterization of its homologues revealed that specific binding of arrestin to the active phosphorylated forms of the great majority of GPCRs stops their G protein-mediated signaling. Arrestins are average sized ~45 kDa proteins with the fold shared with (and probably inherited from) proteins involved in vesicle trafficking. Arrestin family is fairly small: vertebrates express four subtypes, whereas other branches of the animal kingdom have even fewer different arrestins. Yet these few arrestins not only bind hundreds of GPCR subtypes expressed in virtually all animals but also interact with dozens of non-receptor-signaling proteins. Some of these interactions occur regardless of receptor binding, some are promoted by it, while others are precluded or suppressed by GPCR interaction. This places arrestins at an important intersection of signaling pathways in the cell where external and internal inputs are integrated into coherent behavior. This volume describes our current understanding of the biological role of visual and nonvisual arrestins in different cells and tissues, focusing on the mechanisms of arrestin-mediated regulation of GPCRs and non-receptor-signaling proteins in health and disease. This book covers a wide range of arrestin functions, emphasizing therapeutic potential of targeting arrestin interactions with individual partners. Arrestins are ultimate scaffolds: they organize multiprotein signaling complexes and localize them to particular cellular compartments. Everything arrestins do is mediated by protein–protein interactions. Since highly regulated protein–protein interactions underlie most vital cell functions, arrestins are a perfect proving ground for designing novel protein-based therapeutic tools to channel cell signaling in the desired direction.

Nashville, TN — Vsevolod V. Gurevich

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Part II Arrestin Interactions with Non-receptor Partners and Roles in Cell Signaling

Therapeutic Potential of Small Molecules and Engineered Proteins

Eugenia V. Gurevich and Vsevolod V. Gurevich

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Abstract Virtually all currently used therapeutic agents are small molecules, largely because the development and delivery of small molecule drugs is relatively straightforward. Small molecules have serious limitations: drugs of this type can be fairly good enzyme inhibitors, receptor ligands, or allosteric modulators. However, most cellular functions are mediated by protein interactions with other proteins, and targeting protein–protein interactions by small molecules presents challenges that are unlikely to be overcome with these compounds as the only tools. Recent advances in gene delivery techniques and characterization of cell type-specific promoters open the prospect of using reengineered signaling-biased proteins as next-generation therapeutics. The first steps in targeted engineering of proteins with desired functional characteristics look very promising. As quintessential scaffolds that act strictly via interactions with other proteins in the cell, arrestins represent a perfect model for the development of these novel therapeutic agents with enormous potential: custom-designed signaling proteins will allow us to tell the cell what to do and when to do it in a way it cannot disobey.

Keywords Drugs • Small molecules • Enzyme inhibitors • Receptor ligands • Signaling scaffolds • Protein-based therapeutics

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1 Direct Action Drugs: Ligands and Inhibitors

Virtually all traditional drugs, as well as >90 % of therapeutics currently marketed are small molecules (Hopkins and Groom 2002). To be clinically useful, a drug has to be fairly selective, which means that it must bind its target with relatively high affinity, with K_D in the nanomolar range or better. There is direct relationship between the energy of the interaction and the K_D: $\Delta G^0 = -RT\ln(1/K_D)$, where ΔG^0 is change in free energy due to interaction, R is gas constant (1.99 cal/mol degree), and T is temperature (in degrees Kelvin). By virtue of its size a small molecule has few chemical moieties that can engage its target. With very few exceptions reasonably high affinity is only achieved when the target protein "envelopes" the drug, i.e., only cavities or deep grooves in any protein can be successfully targeted. This determines severe thermodynamic limitations to what a small molecule can do. Indeed, among targets of marketed drugs about half are enzymes with a deep catalytic cleft where the drug binds, and most of the rest are receptors, ion channels, or transporters equipped with deep cavities where drugs interact (Hopkins and Groom 2002). Since any nonsubstrate that binds at the catalytic cleft of an enzyme acts as an inhibitor, virtually all enzyme-targeting drugs are inhibitors (Imming et al. 2006). The same is true for transporters. Receptors offer wider range of possibilities despite these limitations: a drug binding in the same cavity where natural ligand binds can be an activator (agonist), neutral antagonist, or an inverse agonist suppressing constitutive activity of the receptor, all competing for the same binding site (Imming et al. 2006). Drugs with all of these modalities are used, well-known examples being beta-blockers (antagonists) used to treat heart diseases and beta-agonists used in asthma.

The key drawbacks of conventional small molecule therapeutics are that they are essentially "one-trick ponies" that can do only one thing and that they keep doing it regardless of the physiological state of the patient, because they are not equipped to receive feedback from the body. For example, if you take a beta-blocker for your heart condition, it will keep blocking beta-adrenergic receptors when you are sitting and using relatively little energy and therefore needing fairly slow heart rate, as well as when you are running and using a lot more energy and oxygen, which requires harder work from the heart to provide increased blood flow. In addition, beta-blocker will block beta-adrenergic receptors in other tissues, which can cause side effects. That is why most drugs come with numerous warnings telling you what to do and not to do after taking the drug, describing various possible side effects, and advising you to stop taking the drug if these unwanted effects are too strong.

There is one area where small molecules are and will likely remain the best possible therapeutic tools: fighting parasites, such as bacteria, fungi, and viruses. Small molecule inhibitors are very effective as antimicrobials because they target enzymes performing biochemical reactions that we don't have, such as building and maintaining cell wall. The most effective antibiotics inhibit enzymes involved in cell wall construction (e.g., penicillin and the whole family of its derivatives) or something else specific for the bacteria, like their ribosomes that are very different

from eukaryotic ones. RNA viruses can be selectively targeted via their reverse transcriptases, as our only enzyme in this class, telomerase, is quite different, and so on. However, when our own proteins need to be regulated for therapeutic purposes, "single-mindedness" of enzyme inhibitors or receptor ligands, as well as their unresponsiveness to the signals sent by the rest of the body becomes a huge liability.

2 Allosteric Modulators: Greater Sophistication

Small molecules have several obvious advantages. First, new small molecule drugs targeting the enzyme or receptor of interest can be devised using well-established procedures (Segall 2012). New compounds with therapeutic potential can be created by generation of new derivatives of known compounds and then selection of the most potent and specific among them. Alternatively, completely new compounds targeting a particular protein can be identified by high-throughput screening of widely available huge chemical libraries and then the same process of generation of derivatives and selection can be applied (Mayr and Bojanic 2009). Both approaches are conceptually straightforward, although quite expensive. Second, it is also fairly well known which chemical groups in putative drugs should be avoided to prevent poor absorption in the gut or rapid metabolism, so this part of drug development also does not require any intellectual breakthroughs, only more funding. Therefore, it was natural that the first attempts to overcome some of the limitations of conventional drugs focused on small molecules.

Receptors are usually medium-sized proteins, where endogenous agonists and conventional orthosteric ligands, all interacting with the same site, that are used as drugs occupy only a small area. The binding of compounds to other parts of the receptor can enhance or reduce activating effect of the agonist, thereby modulating the signaling (Luttrell and Kenakin 2011). To a certain extent, the development of positive and negative allosteric modulators of G protein-coupled receptors (GPCRs) solved one key problem of small molecules. Modulators only act in conjunction with endogenous agonists, decreasing or increasing their action, but remain essentially inactive in its absence (Kenakin and Miller 2010). Thus, the effect of modulators depends on the physiological state of the patient, which makes them superior to conventional orthosteric ligands. This explains rapid expansion of research in this area.

However, even allosteric modulators share quite a few limitations of conventional drugs. First, each of these molecules is designed to do only one thing: it targets an individual receptor (and is carefully selected for this narrow specificity). Second, the only feedback the allosteric modulators respond to is the level of endogenous orthosteric ligand. In addition, the strongest positive modulators at higher concentrations act as allosteric agonists, stimulating the signaling even in the absence of endogenous ligands (Kenakin 2010). Thus, considering the complexity of biological systems, the limited set of functional capabilities of any small molecule remains an unavoidable disadvantage of this approach.

3 Protein-Based Therapeutics: Challenges and Potential

It is widely known that virtually all vital aspects of cellular behavior, such as adhesion, migration, proliferation, and cell death by apoptosis or other mechanisms, are mediated and regulated via interactions of proteins with each other (Elowitz and Lim 2010). Most extracellular signals exert their action by promoting or disrupting interactions of particular proteins in the cell. For example, in case of GPCRs, which are targeted by >30 % of clinically used drugs (Hopkins and Groom 2002), the agonists promote receptor interactions with heterotrimeric G proteins (Samama et al. 1993), then GPCR kinases (GRKs) (Gurevich et al. 2012), and then arrestins (Gurevich and Gurevich 2006). Receptor-dependent activation of G proteins induces dissociation of their α- and βγ-subunits, promoting their interactions with various effector proteins (Dessauer et al. 1996). Receptor-bound arrestins bind clathrin and AP2 (Goodman et al. 1996; Laporte et al. 1999), which triggers GPCR internalization via coated pits, and interact with numerous other proteins, initiating the second round of signaling (Hanson et al. 2006; Xiao et al. 2007). Chains of sequential protein–protein interactions underlie every signaling pathway in the cell.

Therefore, the ability to selectively disrupt or enhance individual protein–protein interactions would give us an unprecedented leverage over the cell, essentially allowing us to tell the cell what to do and when to do it in a language it understands (Gurevich and Gurevich 2012). This will be hugely advantageous scientifically, giving us powerful tools to elucidate the intricacies of cell signaling, which is arguably the greatest current challenge in biological research. This will also pave the way to devising conceptually novel therapeutic approaches with potential to actually cure many congenital and acquired diseases, in contrast to just managing the symptoms, which is the best we can do now in case of asthma, diabetes, depression, mental disorders, heart disease, Parkinson's, Alzheimer's, retinal degeneration, etc.

However, protein–protein interactions are virtually never targeted for therapeutic purposes. Naturally, this is not an oversight: there are real difficulties in targeting these interactions with small molecules, which currently predominate as therapeutic tools. First, protein elements mediating the interaction are very rarely mapped with necessary precision to be targeted, or in most cases are simply unknown (Gurevich and Gurevich 2010). Second, the elements involved are often unstructured ("intrinsically disordered"), and only assume final fold upon interaction, with the help of the binding partner. This coupled folding and binding is sometimes referred to as "fly-casting mechanism" (Shoemaker et al. 2000; Sugase et al. 2007). It is currently impossible to design a small molecule targeting a disordered polypeptide. Most importantly, even when the interacting elements are identified and well ordered with known three-dimensional structure, the interactions are mediated by relatively flat protein surfaces, which do not bind small molecules with high enough affinity (in contrast to deep grooves, like the active sites of most enzymes or ligand-binding sites of receptors). These surfaces are usually also too large [>2,000 $Å^2$ (Jones and

Thornton 1995)] to be significantly modified by a small molecule. These structural limitations suggest that it is highly unlikely that small molecules selectively targeting most individual protein–protein interactions will ever be developed. Last, but not least, every intervention with small molecules attempted so far aimed at disrupting protein–protein interactions (Thiel et al. 2012), whereas it is equally likely that selective strengthening of some of them will be of high scientific and therapeutic value.

The most realistic way of modulating protein–protein interactions in a desired manner is to rely on proteins themselves. By introducing into the cell a protein with modified signaling properties we can affect cell behavior as we like. Cancer cells represent one obvious target: if we could tell them to stop proliferating, that would solve the problem. Another obvious target is dying neurons in neurodegenerative diseases: if we could tell them to stay alive in a way they cannot disobey, we would have a cure. Biological function of signaling proteins is to deliver messages. Thus, we need to learn how to create our own messengers to deliver signals we want and/or to override the signals we disagree with that the cell receives from other sources.

Gene delivery to targeted cell types in humans is no longer science fiction. The development of viral and nonviral gene delivery systems (Bartel et al. 2012; Nguyen and Szoka 2012) and identification of promoters driving the expression in cell types of choice is proceeding at a rapid pace. Recent success of three gene therapy clinical trials where correct RPE65 gene was delivered to the pigment epithelium of Leber's congenital amaurosis patients carrying loss-of-function mutations in this protein (Cideciyan et al. 2008; Hauswirth et al. 2008; Maguire et al. 2008; Bainbridge et al. 2008) demonstrate that gene delivery methods are ready for use today, not in the distant future (Cideciyan 2010; Cao et al. 2011). The elucidation of fine molecular mechanisms of the function of every signaling protein would allow us to design signaling-biased mutants worth delivering by these sophisticated methods. It is particularly important to elucidate general principles of protein–protein interactions and the functional connections within cellular signaling networks to construct custom-designed signaling proteins with the functional characteristics we want and other protein-based molecular tools to tell cells what to do in a way they cannot ignore.

4 Signaling-Biased Arrestins as a Model

In most cases what we need to do for therapeutic purposes is selectively enhance or reduce only one interaction of a particular signaling protein out of a dozen or more it is normally engaged in. Arrestin proteins appear to be a perfect model to test-drive this approach for several reasons. First, arrestins are not enzymes or receptors with a binding pocket that can be targeted by small molecules. Arrestins are classical signaling scaffolds: everything they do in the cell is mediated by their interactions with other proteins (Gurevich and Gurevich 2003; DeWire et al. 2007).

That is why currently there are no ways to affect their functions by small molecules, and it is highly unlikely that any drugs suitable for this purpose will ever be developed. Second, mammals express only two nonvisual arrestins, arrestin-2 (a.k.a. β-arrestin1)[1] and arrestin-3 (a.k.a. β-arrestin2), each interacting with hundreds of different G protein-coupled receptors (GPCRs) and dozens of other signaling proteins (Hanson et al. 2006; Xiao et al. 2007). Third, arrestins are ubiquitous signaling regulators in the cell, involved in multiple pathways, including several that directly regulate cell fate via pro-survival or pro-apoptotic signaling (Gurevich and Gurevich 2010, 2012). This makes arrestins convenient ubiquitously expressed tools for modulating cell behavior.

Structurally, arrestins are characterized better than most signaling scaffolds. Crystal structures of all four vertebrate arrestins have been solved (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011), as well as the structures of arrestin-2 complexes with the interacting elements of clathrin (ter Haar et al. 2000; Kang et al. 2009). Moreover, extensive structure–function studies of arrestin family members revealed the function of numerous amino acid side chains in these relatively small 40–45 kDa proteins [reviewed in Gurevich and Gurevich (2004, 2006) and Gurevich et al. (2011)]. The dynamics of arrestin molecule in solution was studied by a variety of methods, including H/D exchange (Ohguro et al. 1994; Carter et al. 2005), site-directed spin labeling and electronic paramagnetic resonance (EPR) (Hanson et al. 2006, 2007a, b, 2008; Vishnivetskiy et al. 2010, 2011), and nuclear magnetic resonance (NMR) with $^{13}C/^{15}N$ labeled arrestin (Zhuang et al. 2010, 2013). Even though the structure of the arrestin–receptor complex still remains to be solved, receptor binding-induced conformational changes in arrestins were recently characterized by intramolecular distance measurements using double electron–electron resonance (Kim et al. 2012). The same mechanism of arrestin activation by receptor-attached phosphates was shown to operate in all arrestins (Gurevich and Benovic 1995, 1997; Gurevich 1998; Kovoor et al. 1999; Vishnivetskiy et al. 1999; Celver et al. 2002; Sutton et al. 2005). This uniformity was further supported by recently solved structures of the arrestin-2 with C-terminal deletion (Kovoor et al. 1999) in complex with multi-phosphorylated peptide representing the C terminus of the V2 vasopressin receptor (Shukla et al. 2013) and of similarly truncated short splice variant of arrestin-1, p44 (Kim et al. 2013). Both structures, which indicate likely direction of the receptor binding-induced conformational changes, representing arrestins somewhere between basal and receptor-bound state, turned out to be remarkably similar (Kim et al. 2013; Shukla et al. 2013).

The feasibility of structure-based redesign of arrestins to generate mutants with functional characteristics changed in desired direction has already been

[1] Different systems of arrestin names are used in the field and in this book. We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called "*arrestin 3*" in the HUGO database).

demonstrated. Based on the mechanism of arrestin activation by receptor-attached phosphates, the first signaling-biased arrestin mutants that bind with high affinity active unphosphorylated GPCRs were constructed (Gurevich and Benovic 1995, 1997; Gurevich et al. 1997; Gurevich 1998; Kovoor et al. 1999; Celver et al. 2002; Vishnivetskiy et al. 2013a, b). These enhanced arrestins were shown to quench signaling by unphosphorylated receptors in biochemical experiments with purified proteins in vitro (Gray-Keller et al. 1997), in intact cells (Kovoor et al. 1999; Celver et al. 2002), and in transgenic animals in vivo (Song et al. 2009). Enhanced arrestin mutants and their therapeutic potential are discussed in Chap. 7. Receptor-binding surface of arrestins was mapped by several groups, all of which identified multiple residues on the concave side of both arrestin domains as the receptor "footprint." The agreement on this point is rather remarkable, considering wide variety of methods used: H/D exchange (Ohguro et al. 1994), peptide competition (Pulvermuller et al. 2000), element swapping (Gurevich et al. 1993, 1995; Vishnivetskiy et al. 2004), epitope insertion (Dinculescu et al. 2002), site-directed mutagenesis (Hanson and Gurevich 2006; Vishnivetskiy et al. 2011), site-directed spin labeling/EPR (Hanson et al. 2006; Vishnivetskiy et al. 2010, 2011; Kim et al. 2012), and NMR (Zhuang et al. 2013). The finding that very few residues on this extensive surface largely determine receptor specificity (Vishnivetskiy et al. 2011) was unexpected, but entirely welcome. The very first attempt of targeted mutagenesis of identified receptor-discriminator residues yielded versions of inherently promiscuous arrestin-3 with >50-fold preference for some GPCRs over others (Gimenez et al. 2012). The prospects of constructing arrestins specifically targeting groups of receptors or even individual GPCRs are discussed in Chap. 8.

We are approaching the limits of what can be achieved in a complex living organism with small molecules, suggesting that more sophisticated tools are needed. Custom-designed signaling proteins with special functional characteristics are the "smarter" tools we need that along with regulatory RNAs (that also require gene delivery) will likely become next-generation therapeutics. Using reengineered proteins we can manipulate cell signaling in ways that cannot be achieved by other means. Targeted mutations change protein–protein interactions that due to their structural properties most likely will never be successfully targeted by small molecules. In contrast to small molecules that have a single function and do not respond to the physiological state of the patient, proteins with targeted modifications will remain sensitive to normal feedback mechanisms operating in the cell. This minimizes the chances of severe adverse side effects, which arguably doomed more conventional drugs than any other issue.

Critical roles that arrestin proteins play in many biological processes make them a perfect target to develop and test new approaches of manipulating cell signaling for research and therapeutic purposes. Numerous arrestin functions and their structural basis are discussed in this book. These include the mechanisms of receptor binding (Chap. 2), the action of arrestin-biased GPCR agonists (Chap. 3), as well as specific functions of visual subtypes (Chaps. 4–6), the possibility of compensating for the lack of receptor phosphorylation with enhanced arrestins (Chap. 7) and creating mutant forms of nonvisual arrestins to target

specific GPCRs (Chap. 8). In addition to hundreds of GPCRs, arrestins interact with a variety of other proteins (Gurevich and Gurevich 2006; DeWire et al. 2007). Identification of arrestin elements engaging non-receptor partners enabled the construction of mutants where one particular function was disabled, leaving the others virtually intact (Kim and Benovic 2002; Meng et al. 2009; Coffa et al. 2011; Kim et al. 2011; Seo et al. 2011; Breitman et al. 2012). The mechanisms of clathrin and AP2 binding and properties of arrestins lacking these functional modalities are discussed in Chap. 9. The role of arrestins in protein ubiquitination and deunbiquitination are discussed in Chap. 10. The elements involved in self-association of visual and nonvisual arrestins and characteristics of constitutively monomeric mutants are discussed in Chap. 11. Several chapters discuss the role of arrestins in the activation of MAP kinases ERK1/2 (Chap. 12), JNK1/2/3 (Chap. 13), and p38 (Chap. 14). This book also discusses arrestin roles in a variety of other biological processes, such as regulation and localization of PDE (Chap. 15), programmed cell death (Chap. 16), cell motility (Chap. 17), infectious diseases and host–pathogen interactions (Chap. 18), regulation of small GTPases (Chap. 19), airway epithelium and asthma (Chap. 20), cancer (Chap. 21), as well as pain and anesthesia (Chap. 22). Despite enormous breadth, even this volume is not exactly comprehensive, but it gives the reader an idea of the variety of biological roles played by a small family of four vertebrate arrestins.

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Part I
Arrestins and Regulation of G Protein-Coupled Receptors

Arrestin Interactions with G Protein-Coupled Receptors

Martin J. Lohse and Carsten Hoffmann

Contents

Abstract G-protein-coupled receptors (GPCRs) are the primary interaction partners for arrestins. The visual arrestins, arrestin1 and arrestin4, physiologically bind to only very few receptors, i.e., rhodopsin and the color opsins, respectively. In contrast, the ubiquitously expressed nonvisual variants β-arrestin1 and 2 bind to a large number of receptors in a fairly nonspecific manner. This binding requires two

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triggers, agonist activation and receptor phosphorylation by a G-protein-coupled receptor kinase (GRK). These two triggers are mediated by two different regions of the arrestins, the "phosphorylation sensor" in the core of the protein and a less well-defined "activation sensor." Binding appears to occur mostly in a 1:1 stoichiometry, involving the N-terminal domain of GPCRs, but in addition a second GPCR may loosely bind to the C-terminal domain when active receptors are abundant.

Arrestin binding initially uncouples GPCRs from their G-proteins. It stabilizes receptors in an active conformation and also induces a conformational change in the arrestins that involves a rotation of the two domains relative to each other plus changes in the polar core. This conformational change appears to permit the interaction with further downstream proteins. The latter interaction, demonstrated mostly for β-arrestins, triggers receptor internalization as well as a number of nonclassical signaling pathways.

Open questions concern the exact stoichiometry of the interaction, possible specificity with regard to the type of agonist and of GRK involved, selective regulation of downstream signaling (=biased signaling), and the options to use these mechanisms as therapeutic targets.

Keywords Arrestin • β-arrestin • G-protein-coupled receptor (GPCR) • G-protein-coupled receptor kinase (GRK) • Conformational change • Bioluminescence resonance energy transfer (BRET) • Fluorescence resonance energy transfer (FRET) • Biased signaling

1 Introduction

Arrestins are a small family of only four homologous proteins that play important and very versatile roles in the signaling by G protein-coupled receptors (GPCR). They bind to these receptors in a highly regulated manner and through this binding impair some signaling pathways, while at the same time they promote numerous other cellular signals and, in many instances, also aid in receptor internalization and trafficking.

Visual arrestin or arrestin1, the first arrestin protein to be discovered and characterized, had been known long before its role in signaling by rhodopsin, a prototypical G protein-coupled receptor, became apparent. In fact, it was the immunogenicity of what was then named *S-antigen* (its first name, for soluble antigen) and its role in causing uveitis (a form of inflammation of the eye) that brought this protein to the limelight. In the 1980s, through the pioneering work of Hermann Kühn, it became apparent that this *48 kDa protein* (its second name) was involved in signal transduction. It was found to bind to rhodopsin after light activation and to impair rhodopsin's signaling to G proteins and their downstream effectors, cGMP phosphodiesterases. Based on this "arresting" function, but

assuming a different mechanism, i.e., acting directly on the phosphodiesterase, Ralph Zuckerman and colleagues proposed its third name, *arrestin*. Structural and sequence similarities then led by homology to the discovery of the non-visual arrestins (=*arrestin2* and arrestin*3*), which were initially discovered as signaling inhibitors of activated β-adrenergic receptors and were, hence, called *β-arrestin1* and *2*. Finally, a fourth member of this group preferentially expressed in retinal cones was identified by homology cloning and was termed *X-arrestin*, *cone arrestin*, or *arrestin4* (see chapter "Therapeutic Potential of Small Molecules and Engineered Proteins" for the description of two systems of arrestin names).

Through a large number of investigations, it became apparent that these four proteins shared the ability to interact with the active and phosphorylated form of GPCRs, but that they differed in terms of their expression patterns, their specificity for different GPCRs, and their functional effects. These functional effects are elicited via their interaction both with GPCRs and with downstream proteins. Interactions with the receptors first trigger a shutoff of "classic" G protein-dependent signaling, while the latter proteins direct receptor internalization and at the same time trigger "nonclassical" signaling events.

The X-ray structures of all four arrestins have been solved in the last 15 years. They are all remarkably similar and show two concave domains of antiparallel β-sheets connected through a hinge region and the polar core (see chapter "Enhanced Phosphorylation-Independent Arrestins and Gene Therapy," Fig. 3). While phosphorylated receptors bind to the concave sides of the β-sheet domains, the convex sides provide ample space for the docking of many other proteins that can mediate downstream functions.

2 Discovery of Arrestins

2.1 Visual Arrestin (Arrestin1)

Visual arrestin is a soluble 48 kDa protein that is essentially exclusively expressed in the retina, more specifically the retinal rods (and also in cones). When it was first purified from retina and characterized in the 1970s, this was for entirely different reasons: it had been known since the early twentieth century that antigenic substances are present in the eye, when Elschnig (1910) proposed that autoimmune reactions played a causal role in sympathic ophthalmia. In the mid-1960s, it was shown that the retina contains an antigen that can cause uveitis, an autoimmune inflammation of the eye (Wacker and Lipton 1965), and in the mid-1970s, two groups succeeded in isolating the responsible antigen, which because of its soluble character was called S-antigen (Dorey and Faure 1977; Wacker et al. 1977; Wacker 1991).

At about the same time, Hermann Kühn had characterized the light-dependent phosphorylation of rhodopsin, both in vitro (Kühn and Dreyer 1972) and in vivo

(Kühn 1974), and had subsequently observed that not only the responsible specific kinase, hence termed rhodopsin kinase (modern systematic name GRK1, see below), but also additional retinal proteins bound in a light-dependent manner to rhodopsin; these proteins included prominently a 48 kDa protein (Kühn 1978). Subsequent studies by his team showed that this binding was greatly increased not only by light but also by the (again light-dependent) phosphorylation by rhodopsin kinase—indicating that there were two interconnected but presumably independently acting triggers for this binding process (Kühn 1984). In collaboration with the teams of Jean-Pierre Faure and Marc Chabre, they succeeded in showing that their 48 kDa protein and the previously identified S-antigen were in fact the same protein (Pfister et al. 1985).

Not much later it became apparent that binding of this 48 kDa protein interfered with the signaling function from rhodopsin to the effector cGMP phosphodiesterase, which cleaves cGMP to GMP (Miki et al. 1973, 1975; Fung et al. 1981) and thereby causes closure of cGMP-gated ion channels that are normally held open by cytosolic cGMP (Matesic and Liebman 1987). Two alternative hypotheses were initially developed how this inhibitory function might be exerted. Ralph Zuckerman and colleagues proposed that the 48 kDa protein directly inhibited the phosphodiesterase, mostly on the basis of kinetic findings, i.e., a very rapid turnoff of cGMP hydrolysis and competition between the 48 kDa protein and the α-subunit of rhodopsin's cognate G protein, transducin, which activates the phosphodiesterase (Zuckerman and Cheasty 1986). Because the 48 kDa protein rapidly "arrested" phosphodiesterase activation, they suggested calling the 48 kDa protein "arrestin" (Zuckerman et al. 1985)—a name that has stayed with this protein even though the postulated direct inhibition does not appear to be its mechanism of action.

An alternative explanation for the inhibitory effects was proposed by Kühn and colleagues, who observed that the activation of the effector cGMP phosphodiesterase by rhodopsin was quenched, when the 48 kDa protein bound to light-activated, phosphorylated rhodopsin (Wilden et al. 1986). This suggested that it inhibited the signaling function at the level of the communication between rhodopsin and its G protein, transducin (Wilden et al. 1986)—and not at the level of the phosphodiesterase as had been suggested by Zuckerman et al. Together with the light- and phosphorylation-dependent binding described above, this provided a highly specific mode of trigger-dependent inhibition of signaling (Kühn and Wilden 1987). While the mechanism discovered by Kühn and coworkers was rapidly accepted, the name arrestin became generally used for this protein.

The cDNA of arrestin was cloned at the same time by Shinohara et al. (1987) as well as Yamaki et al. (1987) and revealed a hydrophilic protein of 404 amino acids (45.3 kDa). The arrestin sequence was observed to contain several local regions of similarity to the α-subunit of rhodopsin's G protein, transducin, and it was proposed that these regions might enable the protein to bind to rhodopsin. Secondary structure prediction as well as circular dichroism spectroscopy indicated that the protein was primarily composed of β-sheets (Shinohara et al. 1987)—a prediction that turned out to be entirely true when the X-ray structure of visual arrestin and the β-arrestins was solved.

The human arrestin gene was analyzed a few years later (Yamaki et al. 1990). It was found to comprise about 50 kilobase pairs and to contain 16 exons and 15 introns. The length of most exons was less than 100 base pairs, while the introns were much larger. The human sequence was, in addition, reported to code for a 405 amino acid protein.

2.2 Non-visual Arrestins (β-Arrestins)

The remarkable similarities between the rhodopsin and the β-adrenergic (and other similar) receptor systems became apparent in the 1980s, when it became clear that the systems not only consisted of similar functional units—i.e., receptor, heterotrimeric G protein, and effector—but also showed structural similarities (Lefkowitz et al. 1983; Gilman 1984; Hekman et al. 1984; Yamazaki et al. 1985). The structural homologies of the different receptors pertains to the 7-transmembrane helix architecture—known already from bacteriorhodopsin (Henderson and Unwin 1975; Engelman et al. 1982, 1986; Unwin and Henderson 1984)—that became apparent from the primary structure determination of rhodopsin (Ovchinnikov et al. 1982; Ovchinnikov 1982; Hargrave et al. 1983) and the cloning of the cDNAs for rhodopsin and the β_2-adrenergic receptor (Nathans and Hogness 1983, 1984; Dixon et al. 1986). The general principle was further confirmed, when it became apparent that other receptors such as the muscarinic acetylcholine receptor family shared the same structure (Kubo et al. 1986; Bonner et al. 1987; Fukuda et al. 1987; Peralta et al. 1987).

Further similarities were found when it became clear that not only rhodopsin is multiply phosphorylated at its C-terminus in response to its stimulation (see above; Kühn and Dreyer 1972; Wilden and Kühn 1982; Thompson and Findlay 1984) but that a similar stimulation-dependent process occurs for β_2-adrenergic receptors. This agonist-induced phosphorylation seemed to be important for the process of agonist-induced desensitization of these receptors, i.e., a loss of responsiveness upon prolonged or repeated receptor stimulation (Sibley and Lefkowitz 1985; Sibley et al. 1987; Strulovici et al. 1984). The responsible protein kinase was termed β-adrenergic receptor kinase, βARK (Benovic et al. 1986a), and its critical role in so-called homologous (i.e., receptor-specific), rapid receptor desensitization was shown through the use of inhibitors (Lohse et al. 1989, 1990a).

Even before the full purification of β-adrenergic receptor kinase had been achieved (Benovic et al. 1987a), it was shown in collaboration between the Kühn and the Lefkowitz laboratories that this kinase could substitute for rhodopsin kinase and mediate light-dependent phosphorylation of rhodopsin (Benovic et al. 1986b)—further underlining the similarities between the two systems. Interestingly, while the partially purified β-adrenergic receptor kinase impaired signaling by β_2-adrenergic receptors in a manner similar to the inhibitory effects described above for arrestin and rhodopsin, this effect largely disappeared upon full purification, suggesting that an additional component was lost during

purification (Benovic et al. 1987b). Since purified visual arrestin was able to restore this inhibitory effect (Benovic et al. 1987b), it was reasonable to speculate that a homologous protein might exist, which effected the inhibition of β_2-adrenergic receptor signaling triggered by β-adrenergic receptor kinase.

Such a homologous protein was indeed identified by homology cloning of its cDNA, and it was termed β-arrestin, due to its ability to inhibit the signaling of βARK-phosphorylated β_2-adrenergic receptors to their G protein, Gs (Lohse et al. 1990b). It was of similar size (418 amino acids) as visual arrestin and showed 59 % overall identity (75 % similarity) to the latter. The similarities between the two proteins were seen along their entire length, with the greatest diversity occurring along a 15 amino acid stretch in the C-terminal region of β-arrestin (which is not present in arrestin) and in the N- and C-terminal ends.

In direct comparisons between the β_2-adrenergic receptor/Gs and the rhodopsin/transducin systems, both arrestin and β-arrestin were capable of inhibiting signaling by either receptor to a similar extent. However, in terms of the concentrations required to effect this inhibition, a specificity of arrestin vs. β-arrestin by about two orders of magnitude was observed (Lohse et al. 1990b, 1992). This indicates that despite significant homologies the two proteins showed relatively high specificity towards their respective biological systems.

Subsequent studies soon enlarged both the receptor kinase (Benovic et al. 1991; Lorenz et al. 1991) and the arrestin families (Attramadal et al. 1992a, b; Sterne-Marr et al. 1993). Already at the time of cloning of the cDNA of βARK, it had become apparent that this was just one member of a multigene family (Benovic et al. 1989). Today, we count a total of seven receptor kinases, which are now termed G protein-coupled receptor kinases, GRKs. They vary in their tissue expression, their modes of membrane and receptor attachment and their regulatory mechanisms (reviewed in Krupnick and Benovic 1998; Lohse et al. 1996; Pitcher et al. 1998). Two of these kinases are retina specific, i.e., GRK1 (=rhodopsin kinase), which is specific for retinal rods and its receptor rhodopsin, and GRK7, which is specific for retinal cones and phosphorylates the color opsins. In contrast, the other GRKs, most notably GRK2 and 3 (=βARK1 and 2), are not only widely expressed but are also capable of phosphorylating a wide range of GPCRs and also some non-GPCR substrates. While there is a significant receptor selectivity between the retinal (or visual) and the other GRKs, it is not clear how much there is receptor selectivity between the non-retinal GRKs; overall, the promiscuity of these kinases for the many GPCRs that are GRK substrates is quite remarkable. Similarly, it is not clear whether the various GRKs lead to different phosphorylation patterns on the GRKs and whether this affects the functional consequences, including β-arrestin binding (see below).

A second non-visual arrestin, termed β-arrestin2 (Attramadal et al. 1992a, b) or arrestin3 (Sterne-Marr et al 1993) was subsequently discovered, again on the basis of cDNA homology cloning. The 46.3 kDa, 410 amino acid protein encoded by the newly discovered cDNA was more similar to β-arrestin (78 % amino acid identity; 85 % similarity accounting for conservative substitutions) than to visual arrestin (65 % amino acid identity), and it was also widely expressed in the body. Therefore,

it was considered to be a second β-arrestin, i.e., a component of non-visual GPCR systems; hence, it was named β-arrestin2, while the earlier discovered β-arrestin was renamed β-arrestin1 (Attramadal et al. 1992b). The same cDNA was also cloned from a human thyroid cDNA library and the encoded protein proposed to be named THY-ARRX (Rapoport et al. 1992). In terms of function, β-arrestin2 was virtually indistinguishable from β-arrestin1 in its ability to inhibit β_2-adrenergic receptor signaling, while it was weaker than visual arrestin by more than one order of potency in inhibiting rhodopsin signaling (Attramadal et al. 1992b). Only later studies began to reveal differences between the two β-arrestin isoforms.

2.3 Cone Arrestin (X-Arrestin)

Since the expression of visual arrestin had initially been observed to be essentially limited to retinal rods (plus the developmentally related pineal gland; Faure et al. 1984; Abe et al. 1989; Yamaki et al. 1990), it was reasonable to assume the existence of a second visual arrestin that served a similar function in retinal cones. Such a protein was indeed discovered, again via cDNA homology cloning, and termed either cone arrestin (Craft et al. 1994) or X-arrestin, because its gene was mapped to the X-chromosome (Murakami et al. 1993; Sakuma et al. 1998). This 388 amino acid protein had 58 % homology to β-arrestin1 and 50 % homology to visual arrestin. Phylogenetic tree analysis indicated that cone arrestins represent a fourth type of arrestins, the arrestin4 family, which despite their greater similarity to β-arrestin1 versus visual arrestin serve functions only in the retinal cones, i.e., also in the visual system (Craft and Whitmore 1995). The differences between the four proteins were most significant between their C-termini, and the uniqueness of X-arrestin's carboxy-terminal region (from amino acid 353 on) lends support to its distinctness from the other arrestins. Interestingly, in functional binding studies, it had been observed that the C-terminus of visual arrestin is critical for light-dependent binding to rhodopsin (Palczewski et al. 1991a), suggesting that the divergent C-termini might play a role in the relative selectivity of the various arrestins for their receptors.

Immunocytochemistry of this protein has been done with antibodies raised against a unique sequence in its C-terminus, and its expression was compared in double staining experiments with that of the various opsins (Sakuma et al. 1996). These studies indicated its selective expression in cones vs. rods and furthermore showed its co-expression with the red-, green-, and blue-sensitive opsins. These data suggest that it may act as an inhibitor of signaling by the color opsins.

2.4 α-Arrestins

In addition to the two visual and the two non-visual arrestins, a family of proteins has been discovered that may share some of their structural and perhaps also

functional features and that is sometimes referred to as α-arrestins (Aubry et al. 2009; Patwari and Lee 2012). This family of proteins has been predicted to share the overall arrestin-fold structure and to represent the evolutionarily more ancient branch of arrestins, because they can be found already in filamentous fungi, budding yeast, and in protists, which lack β-arrestin homologs (Alvarez 2008). These proteins have been shown to act as E3 ubiquitin ligase recruiting components in yeast (Nikko et al. 2008; Lin et al. 2008) and to play various roles in protein trafficking. Whether this includes trafficking of receptor homologs in yeast, i.e., Ste2 and Ste3, remains to be shown.

Six such α-arrestins have been identified in humans, termed "arrestin domain-containing 1–5," Arrdc1–5, plus "thioredoxin-interacting protein," Txnip (Patwari and Lee 2012). The presence of an arrestin fold (i.e., two curved β-strand sandwich domains connected by a polar core—see below) has been postulated on the basis of the structure of vacuolar protein sorting-associated protein 26A (Vps26), which has been shown to also have this arrestin fold (Shi et al. 2006) and which is more closely related to the α-arrestins than to the visual and β-arrestins (Alvarez 2008). Based on such structural comparisons, more proteins are being recognized that contain an arrestin fold and may be members of this protein "clan" (Aubry and Klein 2013).

Interactions with GPCRs have so far only been reported for arrestin domain-containing 3 (Arrdc3), which has been observed to interact with β-adrenergic receptors and to recruit an E3 ubiquitin ligase to the β_2-adrenergic receptor to mediate its ubiquitination (Nabhan et al. 2010). Furthermore, through a direct interaction with β-adrenergic receptors, Arrdc3 has been suggested to decrease β-adrenergic signaling; conversely, inactivation of Arrdc3 caused increased β-adrenergic signaling, increased energy expenditure and thermogenesis, and ultimately resistance to obesity (Patwari et al. 2011).

Whether such interactions of α-arrestins with GPCRs are a general phenomenon is, however, still largely unclear, as is the question how much they belong to the "true arrestins" (Shenoy and Lefkowitz 2011; Aubry and Klein 2013), and we will therefore not discuss this family of proteins in the subsequent sections of this review.

3 Stimulus-Dependent Interaction of Arrestins with Receptors

3.1 Arrestin-Rhodopsin Model: Phosphorylation and Activation Sensors

Interactions of arrestins with receptors require two different stimuli from the receptor: the active form of the receptor and the prior phosphorylation by a GRK.

Both of these processes had already been elucidated by Hermann Kühn's early research for the arrestin/rhodopsin system (see above). His observation of binding to rhodopsin in a light-dependent manner had in fact been the first implication of an involvement of the 48 kDa protein arrestin in rhodopsin function (Kühn 1978). Because these assay preparations contained both, rhodopsin kinase and arrestin, it was not right away clear that phosphorylation of rhodopsin was an additional prerequisite for arrestin binding—just because under these conditions light-dependent phosphorylation by rhodopsin kinase occurred anyway. However, soon thereafter it became clear that both photoexcitation (=activation) and phosphorylation of rhodopsin represented triggers that greatly enhanced arrestin binding (Kühn et al. 1984).

When visual arrestin and β-arrestin had been cloned, it became possible to express the proteins either in intact cells (Lohse et al. 1990b, 1992; Söhlemann et al. 1995) or by in vitro translation (Gurevich and Benovic 1992, 1993) and to purify them for receptor-binding studies. These studies gave the first indications of receptor specificities and also helped to clarify the roles of receptor activation and phosphorylation for arrestin binding. These studies confirmed that both, activation and phosphorylation of the receptors, were necessary to produce full binding of arrestins to receptors.

The interaction between visual arrestin and rhodopsin appears to be the most specialized and most specific one in several terms (reviewed in Gurevich and Gurevich 2004, 2006): among the various combinations tested, this one has the highest specificity; it is highly sensitive both to agonist stimulation and to GRK-mediated phosphorylation. This mechanism has also been investigated in most detail. These studies have led to the concept that arrestin must contain two types of "sensors" that serve to monitor the corresponding receptor modifications: an "activation sensor" and a "phosphorylation sensor." Both sensors have been investigated extensively with mutagenesis experiments and, more recently, structurally (see below). These studies indicate that the two sensors function largely independently: arrestin binding to phosphorylated light-activated rhodopsin is more than 10 times higher than that to inactive phosphorhodopsin or to active but unphosphorylated rhodopsin, and binding to inactive and unphosphorylated rhodospin is essentially undetectable (Gurevich and Benovic 1993; Gurevich and Gurevich 2006). Thus, receptor activation or phosphorylation alone induced only weak binding of arrestin, whereas the two sensors act in a synergistic fashion. The phosphorylation sensor in arrestin was first identified by mutagenesis (Gurevich and Benovic 1995) and later confirmed in the X-ray structure (Hirsch et al. 1999). A key role was attributed to Arg175 (corresponding to Arg169 in β-arrestin), which was thought to bind to the phosphorylated residues in rhodopsin's C-terminus (but this interaction turned out recently to be indirect—see below). Mutation of this arginine to glutamate resulted in an arrestin (R175E mutant) that binds to activated rhodopsin in a phosphorylation-independent manner (Gray-Keller et al. 1997; Gurevich and Benovic 1997—see below).

On the receptor side, the phosphorylation sensor requires rhodopsin kinase-mediated phosphorylation of rhodopsin's C-terminus. In a detailed study of the

phosphorylation stoichiometry, Vishnivetskiy et al. (2007) observed that one phosphate per rhodopsin did not promote arrestin binding, two resulted in high-affinity binding, and three were required for full arrestin binding, which also appears to involve a conformational change (see below). Since rhodopsin can become multiply phosphorylated (McDowell and Kühn 1977; Wilden and Kühn 1982; Kühn et al. 1984), this indicated a certain "reserve" for this process. However, later experiments suggested that in fact excessive phosphorylation of rhodopsin may be an experimental artifact (Ohguro et al. 1994a) and—if it does occur—even be related to visual disorders (Vishnivetskiy et al. 2007).

Compared to the phosphorylation sensor, which has been mapped with great detail, much less specific sites have been identified for the activation sensor. Identification of the amino acids involved in rhodopsin (or receptor) binding in arrestins have revealed a large number of sites spanning almost the entire two concave sides of arrestin (see below) and indicate either multiple contact points or many and major indirect effects by which amino acids distant from the binding site affect rhodopsin (or receptor) binding (Palczewski et al. 1991a; Gurevich et al. 1994; Kieselbach et al. 1994; Ohguro et al. 1994b; Pulvermüller et al. 2000; Dinculescu et al. 2002; Vishnivetskiy et al. 2004; Hanson et al. 2006; Vishnivetskiy et al. 2013). The structural interpretation of these multiple contact points will be discussed further below. In terms of the definition of the activation sensor, i.e., how arrestin can distinguish between the active and inactive forms of rhodopsin, these studies must be considered still incomplete and may in fact require the determination of the structure of the active complex between an active receptor and an arrestin. However, from detailed spin labeling studies, we know at least that the patterns of interaction between spin-labeled arrestin and inactive or active phosphorhodopsin are different (Hanson et al. 2006).

Once both the activation and the phosphorylation sensors of arrestin have engaged in rhodopsin binding, both partners seem to become structurally affected by this interaction, i.e., both appear to either stabilize (rhodopsin) or change (arrestin) their conformation. Rhodopsin (as well as other receptors) is maintained in its active conformation when bound to its G protein, transducin. This binding is stable during the time of GDP/GTP exchange at the G protein α-subunit. It can be monitored from the fraction of the active, signaling form of rhodopsin, i.e., metarhodopsin II, and this G protein-induced increase has been termed "extra-metarhodopsin II" (Kohl and Hofmann 1987). A similar increase in active metarhodopsin II (or extra-metarhodopsin II) is induced by arrestin (Schleicher et al. 1989). This suggests that binding of arrestin to rhodopsin is at least in this respect similar to binding of (active) G proteins and that both stabilize rhodopsin in its active state—a finding that has similarly been observed for non-visual arrestins and their receptors (see below).

At the same time, the binding process appears to result in a change in the conformation of arrestin, and this conformational change then permits full binding between arrestin and rhodopsin (Gurevich and Gurevich 2004, 2006). The conformational change in arrestin was inferred already in early studies on arrestin-rhodopsin binding (Schleicher et al. 1989) because of the high activation energy

(165 kJ mol^{-1}) of this binding process, which indicated a considerable transient chemical change during the binding process. Subsequent studies indicated in particular that the C-terminus of arrestin is released upon rhodopsin binding (Palczewski et al. 1991a, c; Vishnivetskiy et al. 2002). Interestingly, the activation of this release mechanism does not necessarily require phosphates—other negative charges may suffice, and in fact even completely unrelated polyanions such as heparin are capable of causing release of the C-terminus (Palczewski et al. 1991c; Gurevich et al. 1994). In addition, there must be other conformational changes in arrestin upon rhodopsin binding, since even the binding of a variant of arrestin lacking this C-terminus, called p44 (Smith et al. 1994; Palczewski et al. 1994), has a relatively high activation energy (Pulvermüller et al. 1997). Further support for such a conformational change in arrestins comes from studies on non-visual arrestins (see below).

Collectively, these data have led to a model of arrestin binding to phosphorylated light-activated rhodopsin where initially arrestin "senses" both the active form of rhodopsin and its phosphorylation status and then engages in a high-affinity interaction that involves conformational changes in arrestin and result in stabilization of the active form of rhodopsin (Gurevich and Gurevich 2004, 2006).

3.2 β-Arrestin/β-Adrenergic Receptor Model and Other GPCRs

While the requirements of both, receptor activation and phosphorylation, had been quite clear from early studies of the arrestin/rhodopsin pair, the role of activation for the binding of β-arrestin had initially been less apparent. This was essentially due to the way these experiments had been done, because for the prior GRK phosphorylation step as well as for the activity assays agonists were present in the assays. The phosphorylation step by GRKs had been shown early on to be strictly agonist dependent (Benovic et al. 1986a), and the ability of partial agonists to promote this phosphorylation correlated closely with their ability to produce an intracellular cAMP signal (Benovic et al. 1988). This agonist dependence of GRK phosphorylation appears to be due to two mechanisms; first, active receptors are the natural substrates for GRKs and become good substrates only after adopting the active conformation, and second, active receptors appear to stimulate GRKs (Palczewski et al. 1991b). Furthermore, a second type of agonist dependence became apparent with the observation that GRK2 and 3 (=βARK1 and 2) required in addition the activation of G proteins; this activation releases or positions the Gβγ-subunits so that they can serve as membrane anchors for the kinase, which enhances receptor phosphorylation (Pitcher et al. 1992b). There may be a modest but significant specificity in the ability of various Gβγ-subunits to mediate this effect (Müller et al. 1993). All these mechanisms combine to make phosphorylation of receptors by GRKs strictly agonist dependent.

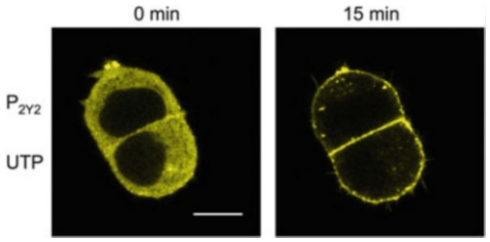

Fig. 1 β-Arrestin2-YFP translocation induced by stimulation of $P2Y_2$ receptor in transiently transfected HEK-293 cells. Images represent the same cells prior to (*left*) and 15 min after (*right*) stimulation with the agonist UTP (100 μM) [Data reproduced with permission from Hoffmann et al. (2008a)]

A requirement for agonists also for β-arrestin binding became obvious only in direct binding assays. Using in vitro translated β-arrestin and several mutants, Gurevich et al. (1993) reported that β-arrestin bound to the activated and phosphorylated form of the M_2 muscarinic acetylcholine receptor with an affinity of 0.5 nM; for β_2-adrenergic receptors the affinity was 0.06–0.14 nM (Gurevich et al. 1995). Similarly, in direct binding assays with purified, reconstituted components, Söhlemann et al. (1995) observed that phosphorylation increased the affinity of β_2-adrenergic receptors for β-arrestin1 from ≈60 nM to only 1.8 nM; in contrast, however, the presence of agonists caused only a modest increase in β-arrestin1 binding. The studies by Söhlemann et al. (1995) also estimated the stoichiometry of binding and came up with a 1:1 ratio of β-arrestin1 to receptors (see below). Overall, the early binding studies revealed that the non-visual β-arrestins appeared to be less discriminating than visual arrestin, with respect both to the activation and the phosphorylation status of the receptors (reviewed in Gurevich and Gurevich 2006).

In addition, these requirements seem not identical in all receptors, and it has been concluded that compared to visual arrestins, where direct phosphate binding is crucial, the interaction of non-visual arrestins with their cognate receptors depends to a lesser extent on phosphate binding and more on the binding to non-phosphorylated receptor elements (Vishnivetskiy et al. 2011). For technical reasons, direct binding studies have been done only for a few receptors, most notably the β_2-adrenergic and the M_2-muscarinic acetylcholine receptors (Gurevich et al. 1993, 1995).

Optical studies in intact cells have greatly aided in understanding the process of β-arrestin binding. These were made initially possible by generating fusions between β-arrestins and variants of the green fluorescent protein (Barak et al. 1997), which enabled the study of agonist-induced translocation of β-arrestins to the cell membrane (Fig. 1), and also allowed a semiquantitative analysis of the binding process (e.g., Vilardaga et al. 2001, 2002; Hoffmann et al. 2008a; Reiner et al. 2009, 2010).

Subsequently, the development of optical energy transfer studies for the interaction between receptors and β-arrestins, FRET (Vilardaga et al. 2003; Krasel

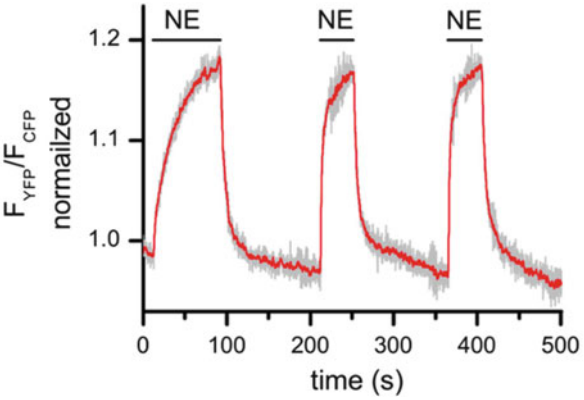

Fig. 2 Agonist-induced FRET between β_2-adrenergic receptor-YFP and β-arrestin2-CFP. Traces of FRET responses (F_{YFP}/F_{CFP}) to superfusion with the agonists in a single HEK cell transiently expressing β_2-adrenergic receptor-YFP and β-arrestin2-CFP. Agonist (300 μM norepinephrine) was present in the superfusion as indicated by *horizontal bars*, showing that the FRET signal was fully dependent on the agonist. Note that the first response is slower than the subsequent ones, indicating that initially receptors need to be phosphorylated by GRKs, and that once they are phosphorylated subsequent interactions with β-arrestin2-CFP are much faster [Data reproduced with permission from Reiner et al. (2010)]

et al. 2005, 2008) and BRET (Bertrand et al. 2002; Pfleger and Eidne 2003; Pfleger et al. 2007; Vrecl et al. 2004), allowed real-time observations of this binding process in intact cells. These studies showed that also for β-arrestins there is a clear requirement for both activation and phosphorylation of receptors to see substantial binding (Vilardaga et al. 2003; Krasel et al. 2005). In fact, in studies with β_2-adrenergic receptors (Krasel et al. 2005, 2008; Reiner et al. 2010), FRET between receptors and β-arrestins required the addition of agonists, and FRET began to disappear as soon as agonist was removed (Fig. 2). Some of these experiments have revealed interesting differences between various agonists acting at one receptor, which have given support to the concepts of distinct active conformations of receptors and biased signaling; these developments will be discussed below.

Similarly, phosphorylation of the receptors was absolutely required, since lack of phosphorylation—either by using a phosphorylation-deficient receptor mutant or by co-transfecting a dominant-negative mutant of GRK2 (GRK2-K220R)—completely abolished the FRET signal. Since GRK-mediated phosphorylation of receptors is relatively slow, it often dominates the kinetics of β-arrestin binding to receptors in intact cells.

The recognition of phosphorylated receptors by β-arrestins is more complex than in the visual system, because of the diversity of recognition sites both in terms of the active conformation and of the phosphorylation. The huge variability of intracellular receptor sequences to which β-arrestins dock suggests that not a specific set of sequences but common structural motifs present in active receptors must define the

β-arrestin docking site. This is a problem that has so far not been solved—neither for the β-arrestins nor for G proteins. A suggestion for a relevant recognition sequence in the second intracellular loop just distal from the conserved DRY motif has come from an analysis of $5HT_{2C}$, β_2-adrenergic, α_{2A}-adrenergic, and NPY_2 receptors (Marion et al. 2006), but it remains to be seen how general these features are.

Heterogeneity in the phosphorylation sites is a second source of complexity, since GRK-mediated phosphorylation occurs not only at the C-termini (as in rhodopsin and the β_2-adrenergic receptor; Dohlman et al. 1987) but also at many other intracellular sites, most notably the third intracellular loop (as in the α_{2A}-adrenergic receptor; Liggett et al. 1992; reviewed in Gurevich and Gurevich 2006). In several instances, β-arrestins even appear to bind to non-phosphorylated receptors (Mukherjee et al. 1999, 2002; Galliera et al. 2004; Jala et al. 2005), and even GRKs themselves can inhibit receptor signaling without phosphorylating the receptors (Dicker et al. 1999).

Combined with mutagenesis of receptors, FRET assays have aided in assessing the phosphorylation requirements of the receptors in more detail. In agreement with observations on rhodopsin discussed above (Vishnivetskiy et al. 2007), phosphorylation of a few residues appears sufficient to promote full interaction with β-arrestins; for example, two such required phosphorylation sites have been identified in the P_2Y_1 receptor (Reiner et al. 2009; Qi et al. 2011), and a cluster of four phosphoserines and threonines is necessary in the β_2-adrenergic receptor (Krasel et al. 2008).

As in the case of rhodopsin, there is evidence for a reciprocal conformational effect that β-arrestins have on the receptors and vice versa. In terms of the receptors, also β-arrestins appear to induce an active state of high agonist affinity, as is typical for G protein-bound receptors (De Lean et al. 1980; Lohse et al. 1984). This active state has been demonstrated by high-affinity agonist binding of the β_2-adrenergic and the M_2 muscarinic acetylcholine receptors (Gurevich et al. 1997) and similarly for the formyl peptide receptor (Key et al. 2001), the neurokinin NK1 receptor (Martini et al. 2002), the glucagon-like peptide-1 receptor (Jorgensen et al. 2005), and the angiotensin II AT_1 receptor (Sanni et al. 2010). In the latter case, there seem to be even slight differences between the receptor states induced by β-arrestin1 vs. β-arrestin2. On the other hand, a conformational change in β-arrestins has been demonstrated by changes in the proteolysis pattern of β-arrestin2 induced by either heparin or phosphopeptides derived from the C-terminus of the V_2 vasopressin receptor (Xiao et al. 2004). Again, these structural changes appear to involve a liberation of the β-arrestin C-terminus (Xiao et al. 2004). Optical techniques have also been used to probe this structural change in β-arrestins in intact cells and a change in BRET between Renilla luciferase (Luc) and the yellow fluorescent protein (YFP) attached to the N- and C-termini of β-arrestin2, respectively (Charest et al. 2005). These changes were observed to occur over a few minutes after stimulation of the V_2 vasopressin receptor and, surprisingly, appeared to be independent of the phosphorylation of the receptor, since they were also seen with a phosphorylation-insensitive β-arrestin2 (R169E, which corresponds to the R175E-

mutant of rhodopsin mentioned above). It was concluded that therefore these changes in BRET probably represent conformational changes promoted by the binding of β-arrestin-interacting proteins, which occurs subsequent to β-arrestin2 binding to the receptors (Charest et al. 2005). Thus, the optical monitoring of the initial conformational change in β-arrestins, which should be induced directly by receptor binding, remains to be achieved.

3.3 Class A and Class B Interactions and Ligand Specificity

While presumably all G protein-coupled receptors bind β-arrestins, there appear to be substantial differences that are related to the specific receptors, to their ability to be phosphorylated by GRKs, to the specific GRK involved in a given situation, and to the ligand that triggers the process. In addition to individual receptor/β-arrestin specificity issues revealed by direct binding assays (see above), there appear to be general patterns that were first identified by Oakley et al. (2000). These patterns allow a subdivision of receptors into two classes of β-arrestin interaction, termed class A and class B. This is a somewhat unfortunate terminology, since the most widely adopted classification schemes of G protein-coupled receptors according to their structures (Bockaert and Pin 1999; Sharman and Mpamhanga 2011; Venkatakrishnan et al. 2013; see also: http://www.iuphar-db.org/DATABASE/GPCRListForward) also use the terminology class A, B, C, etc.

In the context of β-arrestin interactions, this classification proposes on the basis of studies with GFP-tagged arrestin and β-arrestin1 and 2, that class A receptors bind β-arrestin2 with higher affinity than β-arrestin1 and do not interact with visual arrestin, while class B receptors bind both β-arrestins with similarly high affinities and interact also with visual arrestin (Oakley et al. 2000). In this study, class A was represented by β_2- and α_1-adrenergic, μ-opioid, endothelin ET_A, and dopamine D_{1A}-receptors. Class B receptors comprised angiotensin II AT_{1A}-, neurotensin, vasopressin V_2-, thyrotropin-releasing hormone (TRH), and substance P receptors. The key sequence determinants that distinguished class A and class B receptors in this and subsequent studies were distinct GRK phosphorylation sites, most notably in the C-termini of the receptors (Oakley et al. 2000, 2001; Tohgo et al. 2003). Swapping the C-termini between class A and class B receptors changed their β-arrestin binding behavior accordingly.

The patterns of β-arrestin binding defined by these classes appear to affect the functional consequences of β-arrestin binding, such as receptor internalization and recycling as well as signaling to nonclassical pathways like ERKs (see chapter "Arrestin-Dependent Activation of ERK and Src Family Kinases"). These consequences are discussed below. Interestingly, the type of β-arrestin binding appears to be determined not only by the respective receptor but also by the ligand that is used to stimulate the receptor and specific GRK that catalyzes this phosphorylation. This has led to the so-called bar code hypothesis for receptor phosphorylation (Nobles et al. 2011; Liggett 2011; Shenoy and Lefkowitz 2011). This hypothesis

postulates a specific phosphorylation pattern of a given receptor for the different GRKs and receptor ligands. The analysis of the β_2-adrenergic receptor phosphorylation sites upon stimulation with different agonists yielded specific and distinct phosphorylation patterns by either GRK2 or GRK6 and the different ligands. These distinct phosphorylation patterns were then proposed to impart distinct conformations to the recruited β-arrestin, thus regulating its functional activities (Nobles et al. 2011). It has been notoriously difficult to establish the sites of GRK-catalyzed phosphorylation, and only a few successful attempts at complete inventories have been reported to date, which are complicated by a number of technical issues related to in vivo vs. in vitro systems, overexpression, and cell type differences (reviewed by Clark and Rich 2003; see also Godovac-Zimmermann et al. 1999; Soskic et al. 1999; Willets et al. 2003; Tran et al. 2004; Vayttaden et al. 2010). Thus, it will be a great challenge to expand the bar code hypothesis and to determine its consequences for cell signaling (see below).

Ligand specificity for receptor/β-arrestin interactions has also been shown in a few other systems, including some where the differences have also been observed for endogenous agonists. This includes some P2Y receptors, where in the case of the $P2Y_2$ subtype stimulation with ATP induces a preferential interaction with β-arrestin2, whereas stimulation with UTP results in preferential recruitment of β-arrestin1 (see Fig. 3; Hoffmann et al. 2008a; Reiner et al. 2009) and the β_2-adrenergic receptor, where the endogenous agonists adrenaline and noradrenaline appear to differ in their abilities to recruit β-arrestin vs. G proteins (Reiner et al. 2010).

A particularly well-studied example is the μ-opioid receptor, where various ligands can induce very distinct types of signaling, internalization, and desensitization. For this receptor, a large body of evidence suggests that different agonists exhibit bias for G protein activation versus phosphorylation by different kinases and internalization. It has also been suggested that the selectivity of distinct opioid ligands may be due to distinct biochemical receptor forms or conformations, and a number of biased ligands have been proposed to exist. These issues have very recently been covered in a very comprehensive review (Williams et al. 2013) and are also discussed in chapter "Quantifying Biased β-Arrestin Signaling."

4 Functional Effects of the Arrestin/Receptor Interaction

4.1 Termination of G Protein Signaling

The first functional effect that was assigned to arrestins is the quenching of the rhodopsin signal that was observed by Kühn and coworkers (Wilden et al. 1986). This type of inhibition of signaling between a receptor and its G protein was the prevailing function assigned to arrestins for much of the following decade, both for the visual system and for β-arrestin binding to non-visual receptors (Lohse et al. 1990b, Lohse et al. 1992). The process was investigated in much detail for

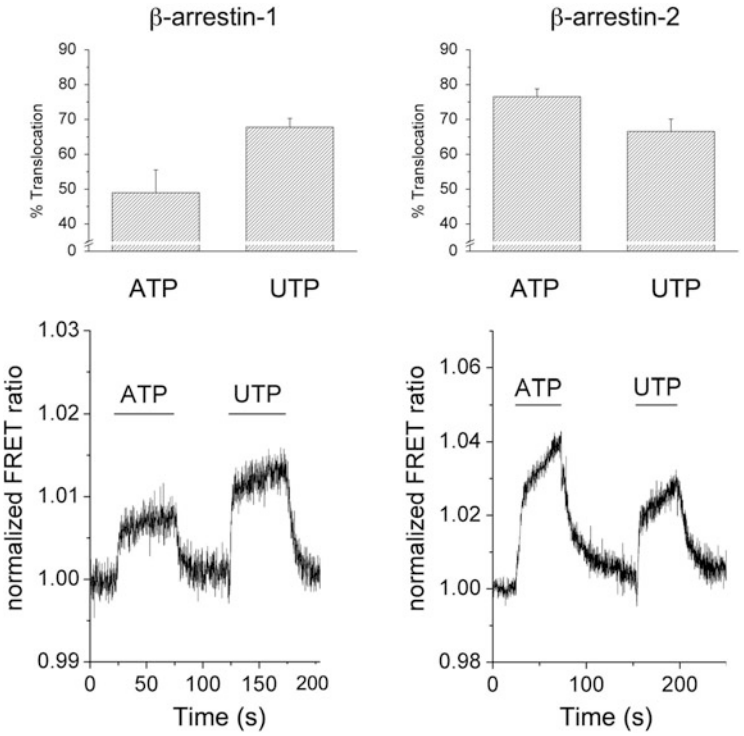

Fig. 3 Agonist (ATP vs. UTP)-selective interactions of $P2Y_2$ receptors with β-arrestin1 and β-arrestin2. The *top panels* show translocation to the cell surface, quantified from images as shown in Fig. 1, quantified as the loss of cytosolic fluorescence. The *bottom row* shows traces of FRET responses (F_{YFP}/F_{CFP}) to superfusion with the agonists in HEK cells transiently expressing $P2Y_2$-YFP and β-arrestin1-Cerulean (*left*) or $P2Y_2$-YFP and β-arrestin2-CFP (*right*). In both experiments, UTP induced stronger translocation for β-arrestin1 than ATP, while ATP induced stronger translocation for β-arrestin2 than UTP [Data reproduced with permission from Hoffmann et al. (2008a)]

the rhodopsin/arrestin system, where it appears to serve two major functions: termination of single photon responses as well as adaptation to various intensities of light (Arshavsky 2002; Ridge et al. 2003; Lamb and Pugh 2004). The wide range of sensitivity of rod outer segments—ranging from single photon detection in the dark to intense sunlight—is a unique feature of the visual system, and arrestin appears to play a major role in this adaptive phenomenon. Since the single photon response of (dark adapted) rods is of a quite defined nature, this means that the process of rhodopsin phosphorylation and subsequent arrestin binding must be tightly controlled. It has been postulated that two types of mechanisms may make sure that this does not simply occur in a stochastic manner (Arshavsky 2002): (a) progressive phosphorylation at multiple sites and (b) feed-forward regulation by release of rhodopsin kinase from the Ca-binding protein recoverin, which occurs when the free Ca concentration is reduced in the course of light-induced activation

(Whitlock and Lamb 1999; Kennedy et al. 2001; Arshavsky 2002). Multiple rhodopsin phosphorylation promotes arrestin binding, with full deactivation speed at three (Vishnivetskiy et al. 2007) or even more (Wilden 1995) phosphates per rhodopsin. This sequence of multiple rhodopsin phosphorylation followed by arrestin binding, together with rapid deactivation of Gt (transducin) by the cGMP phosphodiesterase (Arshavsky and Bownds 1992) and RGS9, is responsible for the rapid shutoff of the single photon response. An additional role for other arrestin variants, in particular the p44 variant, has recently been suggested (Kim et al. 2013), because this variant may (a) exist already in a pre-activated form poised for rhodopsin binding and (b) because of its lower selectivity be already prebound to rhodopsins and, thus, be more easily available than full-length arrestin, which in the dark is largely localized in the inner segment and cell body of the rods and only translocates to the outer segment in response to light (Broekhuyse et al. 1985).

In essence, the other arrestins appear to function in similar switch off mechanisms, but they also show some peculiarities. First cone arrestin (arrestin4) seems to coexist in cones with arrestin1, which actually is also in cones the far predominant arrestin isoform (Nikonov et al. 2008), but it may have functional features that distinguish it from the latter (Gurevich et al. 2011). First, binding of arrestin4 to cone opsins appears to be more transient and of lower affinity (Sutton et al. 2005). This may accelerate the regeneration of the color opsins in cones, which operate mostly in bright light and where both arrestin-independent and arrestin-dependent regeneration after bright light are much faster than in rods (Nikonov et al. 2008). Second, because in contrast to arrestin1 (Schubert et al. 1999), arrestin4 does essentially not di- or oligomerize (Hanson et al. 2008), and since only monomeric arrestins bind to receptors (Hanson et al. 2007b), arrestin4 binding may occur more rapidly, since its binding does not require a prior dissociation step.

For the non-visual arrestins, the β-arrestins, the inhibition of receptor signaling in concert with the GRKs was initially the function, for which a protein was sought (Benovic et al. 1987a, b) and then found (Lohse et al. 1990b). Inhibition of G protein stimulation by β_2-adrenergic receptor and rhodopsin, respectively, was the mechanism used to identify their function and specificity, both with purified components (Lohse et al. 1992) and in intact cells (Pippig et al. 1993). Studies in intact cells defined the sequential action of GRKs (then termed βARKs) and arrestins as the key mechanism of so-called homologous desensitization (i.e., desensitization only of the receptor that was stimulated) and to distinguish it from PKA- and PKC-mediated heterologous (i.e., generalized) desensitization (Lohse et al. 1989, 1990a; Pitcher et al. 1992a; Pippig et al. 1993).

While these studies have firmly established a critical role for β-arrestins in desensitization, i.e., adaptation to signal intensity similar to light adaptation in the visual system, it is less clear whether β-arrestins also play a role in terminating an individual signal. Kinetic measurements suggest that this is possible. For example, the time course of the interaction of β-arrestins with receptors in intact cells is fairly rapid, with half-lives on the order of 5–15 s (Krasel et al. 2005, 2008; Reiner et al. 2010), which is in the same range as G protein deactivation times (by their

GTPase activity) in intact cells (Hein et al. 2005, 2006). Interestingly, the speed of β-arrestin/receptor interaction is much increased when the receptors are pre-phosphorylated by GRKs (Krasel et al. 2005; Reiner et al. 2010; see Fig. 2), suggesting that GRK-mediated phosphorylation is the rate-limiting step, and also that following an initial stimulus (which results in such pre-phosphorylation), receptors might become more sensitive to β-arrestin-mediated inhibition, which might constitute a mechanism of receptor (and synaptic) plasticity (Krasel et al. 2005). These kinetic considerations indicate that a stimulated receptor has only a few seconds at most to transmit its signal to G proteins and downstream signaling proteins, before it is phosphorylated by GRKs and switched off by β-arrestins.

4.2 Receptor Internalization and Trafficking

It was a surprising discovery when independently the groups of Marc Caron (Ferguson et al. 1996) and Jeff Benovic (Goodman et al. 1996) reported that in addition to their effects on receptor/G protein communication, β-arrestins played a major role in receptor internalization, another mechanism that was, at the time, considered a major mechanism of receptor desensitization (Lohse 1993). Both reports found that β-arrestins aided internalization of β_2-adrenergic receptors, in particular of internalization-deficient receptor mutants, and that conversely certain β-arrestin mutants impaired receptor internalization. These effects appeared to be specific for β-arrestins and were not observed for visual arrestin, suggesting major differences between visual arrestins and β-arrestins (Goodman et al. 1996). Mechanistically, the internalization-promoting effects of β-arrestins appeared to be due to a stoichiometric interaction with clathrin, the major structural protein of coated pits, and it was proposed that β-arrestins act as adaptors in receptor-mediated endocytosis via clathrin-coated pits and vesicles (Goodman et al. 1996) (see also chapter "β-Arrestins and G Protein-Coupled Receptor Trafficking"). The critical role of both GRKs and β-arrestins was confirmed by the observation that their kinetics dictate the speed of receptor internalization (Menard et al. 1997).

An interesting aspect of this role was the fact that it had just become appreciated that receptor internalization might be a way of resensitizing receptors (Yu et al. 1993; Pippig et al. 1995). Thus, β-arrestins appeared to play a dual role in regulating receptor sensitivity: they desensitized receptors by preventing their interaction with G proteins, and they resensitized them by promoting internalization, which was followed by dephosphorylation of the receptors (Krueger et al. 1997) and subsequent recycling to the cell surface. The class A vs. class B type of β-arrestin/receptor interaction mentioned above further appears to determine the fate of the receptors: if β-arrestins dissociate more easily (class A), the receptors recycle, whereas receptors with tightly bound β-arrestin (class B) are more frequently targeted to lysosomes for degradation (Oakley et al. 1999, 2000).

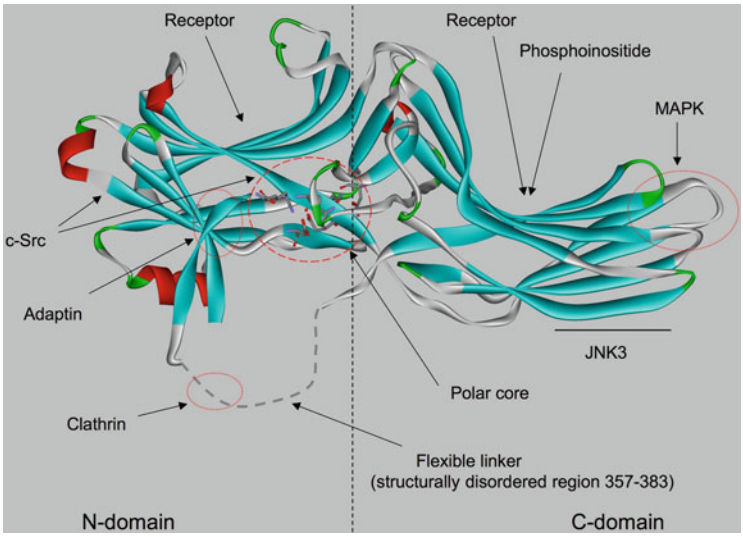

Fig. 4 Tentative assignment of protein interaction sites on β-arrestin. The polar core (D26, R169, K170, D290, D297, and R393) is indicated by the *dashed central circle*. Receptor binding has been shown to occur on multiple sites on the concave sides of β-arrestin. Proposed interaction sites for MAP Kinase, cSrc, JNK3, and phosphoinositides are indicated. The *dashed connecting line* at the *bottom* of β-arrestin indicates a structurally disordered region from amino acid 357–383, which contains the clathrin-binding motif (LIEFD^{380}). The adaptin binding site (DIVFEDFARQR^{395}) is not fully resolved in the crystal structure but indicated by a *small circle*. Data are combined from Lefkowitz and Shenoy (2005) and Kang et al. (2013)

This concept was soon generalized to many more receptors (reviewed by Moore et al. 2007; Shenoy and Lefkowitz 2011; Kang et al. 2013). However, it was also soon realized that receptor internalization occurred not exclusively via this pathway and that it had β-arrestin-dependent as well as β-arrestin-independent components, which varied from one receptor to another (e.g., Zhang et al. 1996; Blaukat et al. 1996; Pals-Rylaarsdam et al. 1997).

These observations indicated that β-arrestins bind not only to receptors but also to other proteins, such as clathrin and β-adaptin, the β-subunit of the clathrin-binding adapter AP2 (Goodman et al. 1996). More recently, it has been reported that the interaction with the internalization machinery involves additional proteins: the small guanosine triphosphatase ARF6 and its guanine nucleotide exchange factor ARNO and the *N*-ethylmaleimide-sensitive fusion protein, NSF (reviewed by Lefkowitz and Shenoy 2005). Very recent data indicate that these functions of β-arrestins are not shared by the so-called α-arrestins (see above) or arrestin domain-containing proteins, putting the latter proteins clearly aside (Han et al. 2013). Taken together, it now appears that β-arrestins bind multiple proteins and that in fact they may be regarded as scaffold proteins that link receptors to a plethora of other cellular proteins (reviewed by Lefkowitz and Shenoy 2005;

Shenoy and Lefkowitz 2005, 2011; Kang et al. 2013). A summary of the most important interaction partners and their tentative binding sites is depicted in Fig. 4.

The role of β-arrestins in receptor internalization and recycling is further complicated by the fact that β-arrestins become multiply modified during this cycle (reviewed by Shenoy and Lefkowitz 2011). First, β-arrestins1 and 2 in the cytosol are usually phosphorylated by ERK-dependent phosphorylation, and they become rapidly dephosphorylated upon agonist-stimulated recruitment to receptors (Lin et al. 1999, 2002). Second, β-arrestins recruit the E3 ubiquitin ligase mdm2, which results in ubiquitination of β-arrestins and results in more stable β-arrestin/receptor complexes, which are then internalized; this ubiquitination (and its functional effects) are reversed by the deubiquitinase USP33 (Shenoy et al. 2009) (see also chapter "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications"). And third, S-nitrosylation of β-arrestin2 enhances its interaction with clathrin and β-adaptin (Ozawa et al. 2008; Lohse and Klenk 2008). And finally, further adapters, such as the sodium/protein exchanger regulatory factor, NHERF1, may regulate the binding of β-arrestins to receptors (Wheeler et al. 2007; Klenk et al. 2010).

4.3 Nonclassical Signaling Pathways

Internalization of receptors does not only remove them from the cell surface and target them to either recycling or degradation, it also triggers new signaling pathways (see chapters "Arrestin-Dependent Activation of ERK and Src Family Kinases," "Arrestin-Dependent Activation of JNK Family Kinases," and "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences"). The scaffolding function of β-arrestins allows them to recruit multiple signaling molecules (reviewed by Lefkowitz and Shenoy 2005; Shenoy and Lefkowitz 2005, 2011). The three major classes are (a) non-receptor tyrosine kinases, such as cSrc, Hck, Fgr, and Yes, which are recruited to various receptors by β-arrestins; (b) components of MAP kinase cascades, both of the module that activates ERK1 and ERK2 (Raf, MEK, ERK) and of the module that activates JNK3 (ASK1, MKK4, JNK3); and (c) the kinases phosphatidylinositol-3-kinase (PI3K) and AKT.

Receptor internalization appears to be closely linked to the activation of these "nonclassical", i.e., G protein-independent, signaling pathways. For example, signaling to MAP kinases appears to occur at the clathrin-coated pits and vesicles (McDonald et al. 2000; DeFea et al. 2000; Luttrell et al. 2001), and it appears to persist as long as the receptors remain bound to β-arrestins, corresponding to the class A/class B types of β-arrestin/receptor interactions (Tohgo et al. 2003).

Temporally, the activation of nonclassical signals follows the G protein-mediated effects, and binding of β-arrestins to the receptors is interposed between these two "signaling waves." A third wave of receptor signaling appears to follow, at least for

some receptors, once they have released their bound β-arrestins (Calebiro et al. 2010). This has been shown at the same time for three different G-coupled receptors, the Gs-coupled parathyroid hormone (Ferrandon et al. 2009) and thyroid stimulating hormone receptors (Calebiro et al. 2009), and the Gi-coupled sphingosine phosphate 1 receptor (Mullershausen et al. 2009). Recently, microscopic images with nanobodies recognizing active forms of β_2-adrenergic receptors and Gs have revealed such active, signaling states of receptors and G proteins on endosomes (Irannejad et al. 2013). β-Arrestin- and dynamin-dependent internalization is required for this type of intracellular signaling. Thus, β-arrestins coordinate a temporally and spatially regulated pattern of receptor-mediated signaling (Lohse and Calebiro 2013): they shut off the first wave of signaling, which consists of activation of G proteins at the cell surface, they trigger a second wave of nonclassical signals (non-receptor tyrosine kinases, MAP kinases, PI3 kinase, and AKT), and they finally permit a third wave of signals, mediated by intracellular activation of G proteins.

4.4 Biased Signaling

The existence of several signaling waves, in particular of G protein-dependent and β-arrestin-dependent signaling, raises the possibility of selective activation of one or the other kind of pathway. Such selective or "biased" signaling (Drake et al. 2008) has become a hot topic over the past years (reviewed in Rajagopal et al. 2011; Reiter et al. 2012; see also chapter "Quantifying Biased β-Arrestin Signaling"). The basic assumption is that the diverse functions of activated receptors may require slightly different active conformations and that such different active conformations might be induced by specific ligands. Indeed, a large number of studies using different full and partial agonist ligands, site-directed mutagenesis, engineered Zn-binding sites locking receptors in distinct conformations, and probes placed in different parts of receptors have provided evidence for distinct active conformations (reviewed in Hoffmann et al. 2008b; Lohse et al. 2008; Seifert and Dove 2009).

For example, studies with engineered Zn-binding sites in the parathyroid hormone receptor have shown differential requirements of G protein activation vs. interactions with GRKs and β-arrestins (Vilardaga et al. 2001), and mutagenesis studies on the same receptor have even allowed a distinction between determinants for β-arrestin binding and for internalization (Vilardaga et al. 2002). More directly, studies probing receptor conformations with fluorescent probes (Nikolaev et al. 2006; Zürn et al. 2009; Mary et al. 2012; Malik et al. 2013) or with hydrogen/deuterium exchange (West et al. 2011) have provided evidence for distinct agonist-activated states. The high conformational flexibility of active receptors, which has recently been observed both in modeling studies (Simpson et al. 2011) as well as experimentally in NMR studies (Nygaard et al. 2013) of β_2-adrenergic receptors, further supports this notion.

Today, there is increasing evidence for biased signaling in many receptor systems. Surprisingly, in most systems it appears that biased ligands generally favor β-arrestin-mediated signaling—perhaps suggesting that natural, endogenous ligands produce only limited activation of β-arrestin-dependent pathways. In some instances, for example, the $P2Y_2$ and the β_2-adrenergic receptor, biased signaling also relates to endogenous agonists (see above). The $P2Y_2$ receptor would be classified as class A receptor when stimulated with ATP, whereas stimulation with UTP would classify the receptor as class B (Hoffmann et al. 2008a). At the β_2-adrenergic receptor, norepinephrine can be classified as Gs-biased when compared to epinephrine (Reiner et al. 2010).

More detailed analyses of the activation mechanisms of angiotensin II AT_1 receptors with resonance energy transfer probes suggest that biased ligands may provoke a "new" active AT_1 receptor entity, which differs in its mode of G protein activation (Saulière et al. 2012). The authors propose that biased agonists do not have to select between effects produced by physiological agonists but may instead stabilize and create a new distinct active pharmacological receptor entity with its own signaling properties. The pharmacological potential of such new ligands is just beginning to be explored. A few interesting examples will be given in the outlook below.

5 Structural Basis of Receptor/Arrestin Interactions

The X-ray structures of all four arrestins have been solved over the past 15 years, some of them independently (reviewed by Gurevich and Gurevich 2013). This began with two crystal structures of visual arrestin (Granzin et al. 1998; Hirsch et al. 1999), followed by structures for β-arrestin1 (Han et al. 2001; Milano et al. 2002), cone arrestin (Sutton et al. 2005), and finally β-arrestin2 (Zhan et al. 2011). Overall, these structures are all remarkably similar, consisting of two concave domains of antiparallel β-sheets connected through a hinge region and the polar core, and a short α-helix on the back of the amino-terminal fold. While receptors bind to the concave sides of the arrestins, the convex sides provide ample space for the docking of many other proteins that mediate downstream functions and play a role in nonclassical signaling pathways (see above).

The polar core is a common element in all arrestin structures, including the central role of Arg175 (or homologous positions), which is stabilized in the inactive structure by a network of charged residues, in particular a salt bridge Arg175–Asp296 (Vishnivetskiy et al. 1999), and which is crucial for the interaction with the negatively charged phosphate residues in phosphorylated receptors (Gurevich and Benovic 1993, 1997). Destruction of the salt bridge by charge reversal mutations on either side (i.e., R175E or D296R) results in arrestins that bind in a phosphorylation-independent manner. Conversely, combination of the two mutations (R175E + D296R) recreates the salt bridge and also phosphorylation-

dependent binding of arrestin to rhodopsin (Vishnivetskiy et al. 1999; Gurevich and Gurevich 2006).

This polar core is stabilized in part by an extended carboxy-terminal tail that locks arrestins in their inactive state (Hirsch et al. 1999). Movement of the C-terminus away from this position is apparently part of the activation mechanism and enables its interactions with partner proteins, for example, the different members of the receptor internalization machinery (see above).

Although the overall structures of all arrestins are so similar, minor differences may help to explain the specific behavior of the different members. The most notable difference is the greater promiscuity of the β-arrestins, which have to interact with many non-visual receptors. This is most pronounced for β-arrestin2, which produces high-affinity interactions with class A as well as class B receptors (see above). In contrast to the other three arrestins, part of the receptor-binding surface in the C-terminal domain of β-arrestin2 fails to form a contiguous β-sheet, which is consistent with increased flexibility (Zhan et al. 2011). Exchange of this region between β-arrestin2 and 1 was found to be correlated with reduced selectivity for activated receptors, consistent with the idea that greater flexibility in the receptor-binding site facilitates accommodation of different receptors.

The stoichiometry of receptor binding to arrestins has been the subject of intensive experimentation, but equally intensive speculation (reviewed by Gurevich et al. 2011; Gurevich and Gurevich 2013). Early in vitro binding studies of β-arrestin to β_2-adrenergic receptors had hinted at a 1:1 ratio (Söhlemann et al. 1995). However, with the realization that—like many other receptors—G protein-coupled receptors can dimerize (Pin et al. 2007; Lohse 2010), and with the crystal structures of arrestins showing two "cups" that might bind one receptor each, 2:1 ratios of receptor/arrestin became popular (e.g., Fotiadis et al. 2006). Experimental data have mostly confirmed the 1:1 model. For example, studies with isolated rhodopsin inserted into nanodiscs (i.e., one molecule of rhodopsin per nanodisc) have shown that a single rhodopsin binds a single arrestin (Tsukamoto et al. 2010; Bayburt et al. 2011)—just as single rhodopsin or other receptor in nanodiscs activates single G proteins (Bayburt et al. 2007; Whorton et al. 2007, 2008). Binding assays with purified arrestin and rhodopsin also indicated a 1:1 stoichiometry, and in vivo in transgenic mice, changes in the expression level of both proteins showed that light-induced arrestin translocation to the rhodopsin-containing compartment was between 80 and 100 % of the molar amount of rhodopsin (Hanson et al. 2007a). Stoichiometric recruitment of arrestin to activated rhodopsin was also found in the *Drosophila* eye (Satoh et al. 2010). These observations agree also with the expression levels of arrestin/rhodopsin in rods, which have been found to be on the order of about 0.8:1 (Strissel et al. 2006; Hanson et al. 2007a; Song et al. 2011).

Docking active receptor molecules to arrestins based on the X-ray structures (Fig. 5)—including those of activated forms described below—illustrates that the receptor-binding surface in arrestins is considerably larger than the cytosolic surface of active receptors (Fig. 6). There are several ways how this discrepancy might be resolved. First, it is possible that some of the residues that appear to be involved in receptor binding do not directly interact with receptors, but that they

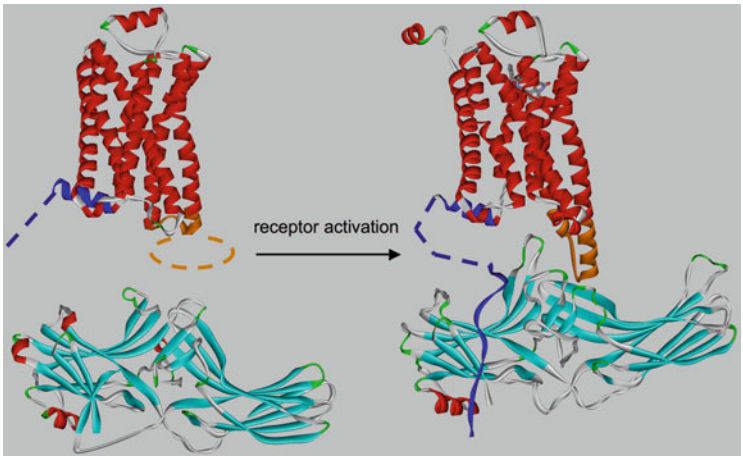

Fig. 5 Proposed scheme of agonist-mediated β-arrestin/receptor interaction. The *left side* of the scheme shows the crystal structures of the inactive β_2-adrenergic receptor bound to carazolol (PDB code 2RH1) and β-arrestin1 in its inactive state (PDB code 1G4M). The *dashed circle* and *line* indicate the third intracellular loop and C-terminus of the β_2-adrenergic receptor, respectively, which were not resolved in the crystal structure. Upon agonist activation, the third intracellular loop of the β_2-adrenergic receptor alters its structural appearance, and the receptor's C-terminus can interact with β-arrestin and release the restrain from the polar core. The *right side* of the scheme shows the active Gs-bound structure of the β_2-adrenergic receptor (PDB code 3SN6) and the active β-arrestin1 structure in complex with a phosphopeptide derived from the V_2-receptor (PDB code 4JQI). The *dashed line* indicates the (uncertain) connection with the C-terminal receptor peptide

affect the binding only indirectly. Second, arrestins might change their conformation in a "clamshell"-like manner and wrap around the cytosolic face of the receptors (Gurevich and Gurevich 2004); this conformational change may be equivalent to the functional effects of arrestin activation that have been discussed above (Palczewski et al. 1991a; Gurevich et al. 1994; Xiao et al. 2004). Third, receptors in the arrestin-bound mode might adopt a different structure, with the cytosolic face even wider than in the G protein-bound state (Rasmussen et al. 2011); evidence quoted above suggests that indeed conformational requirements for G proteins and for arrestins may differ (Vilardaga et al. 2001, 2002; Hoffmann et al. 2008a), which appears to be the basis for biased agonism (Reiter et al. 2012; see above). And fourth, arrestins might bind to two receptors, even though high-affinity binding seems to occur to only one.

In fact, such an alternative model for arrestin/receptor binding has recently been proposed by Sommer, Hofmann, and colleagues (Sommer et al. 2011, 2012) (see chapter "Not Just Signal Shut-Off: The Protective Role of Arrestin1 in Rod Cells"). This model was initially developed on the basis of the observation that in in vitro experiments with rod outer segment membranes, the stoichiometry of arrestin/rhodopsin binding increased with increasing light intensity, from 1:1 to 1:2 (Sommer et al. 2011). It proposes two types of rhodopsin or opsin/arrestin interactions (opsin

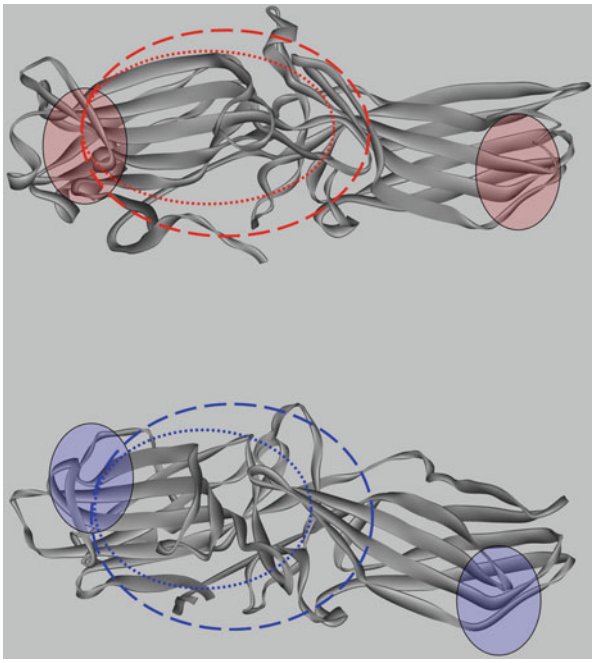

Fig. 6 Spatial requirements for arrestin/receptor interactions, indicating the receptor interaction sites on visual arrestin (*top*) and β-arrestin2 (*bottom*), together with the sizes of the cytosolic faces of rhodopsin (*top*) and the β_2-adrenergic receptor (*bottom*), respectively. The *top panel* represents the structure of visual arrestin (PDB code 1CF1); the size of inactive rhodopsin (*dotted ellipsoid*, PDB code 1F88) and of active opsin (*dashed ellipsoid*, PDB code 3CAP) is projected on the N-terminal domain of arrestin. The *bottom panel* represents the same for the β-arrestin2/β_2-adrenergic receptor pair, showing the structure of β-arrestin2 (PDB code 3P2D) and the size of the inactive (*dotted ellipsoid*, PDB code 2RH1) or the active β_2-adrenergic receptor (*dashed ellipsoid*, PDB code 3SNG). Receptor sizes were determined using DS viewer Pro 5.0. Transparent ellipsoids at the *left* and *right sides* of (β-)arrestin indicate the most distant regions shown to be involved in receptor interactions [taken from Hanson et al. (2006) for arrestin, and from Gimenez et al. (2012) for β-arrestin2]

denotes the protein lacking the covalently bound retinal ligand): a high-affinity interaction with the N-terminal domain and a lower affinity interaction with the C-terminal domain. The high-affinity interaction would be particularly important for the quenching of signaling by the active rhodopsin form, metarhodopsin II. This results in arrestin binding to an asymmetric rhodopsin dimer, where arrestin can stimulate binding of the agonist all-trans-retinal to one of the opsins (Sommer et al. 2012). Such asymmetric binding to receptor dimers has also been proposed for G proteins, where one receptor protomer may bind the α- and the other one the βγ-subunits (Damian et al. 2006; Lohse 2010; Ambrosio and Lohse 2010; Maurice et al. 2011).

So far, there are no structures of a receptor/arrestin complex that might resolve these questions. However, a few recent structural data give some indications how

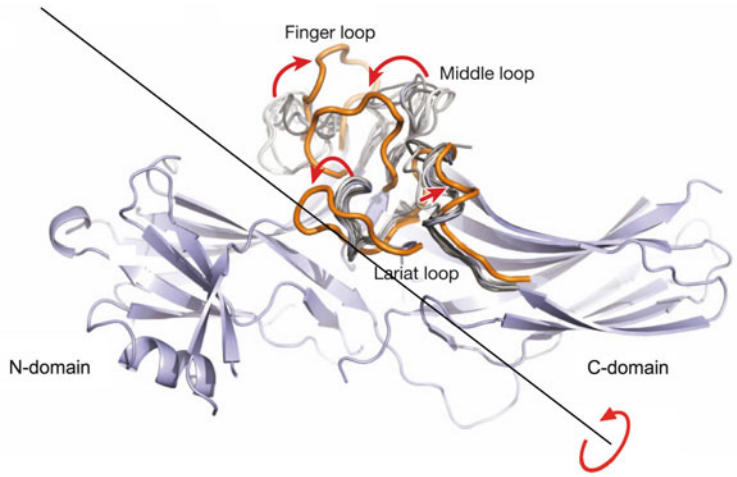

Fig. 7 Structural alterations occurring in β-arrestin1 upon binding of the C-terminal phosphopeptide of the vasopressin V_2 receptor. The figure was modified from Shukla et al. (2013). The *black line* represents the axis of the general 20° rotation of the C- versus the N-terminal domain that was observed upon β-arrestin1 activation. The *arrows* in the central part indicate movements of the finger loop, middle loop, and lariat loop

the binding might occur. First, there is a set of data of double electron-electron resonance (DEER) data on spin-labeled visual arrestin and the changes induced by binding to activated, phosphorylated rhodopsin (Kim et al. 2012b). These data show that the relative position of the N- and C-domains remains largely unchanged, which contradicts the model of a large, "clamshell"-like conformational change. In addition, a number of movements were observed around the polar core, notably of the "finger loop" (amino acids 67–79) and unexpectedly of a loop containing residue 139. The latter movement was subsequently confirmed in mutagenesis experiments (Vishnivetskiy et al. 2013). Several mutants in this loop showed a loss in selectivity for the phosphorylated, activated form of rhodopsin, indicating that the 139-loop stabilizes the inactive conformation of arrestin and reduces its binding to non-preferred forms of rhodopsin.

More recently, two (presumably partially) active X-ray structures of visual arrestin and of β-arrestin1 were reported (Kim et al. 2013; Shukla et al. 2013). The visual arrestin structure (Kim et al. 2013) reported the structure of the pre-activated p44 splice variant (Smith et al. 1994; Pulvermüller et al. 1997), in which the activation of arrestin is mimicked by C-terminal truncation, whereby the stabilization of the polar core by the C-terminus is abolished. An earlier structure of the same protein (Granzin et al. 2012) had revealed only minor changes vs. inactive arrestin compared to the newer structure and may therefore correspond to a largely inactive state of this protein. The β-arrestin1 structure was obtained in complex with a fully phosphorylated 29-amino-acid carboxy-terminal peptide derived from the human V_2 vasopressin receptor; this complex was stabilized with a conformationally selective synthetic antibody fragment (Fab30). It had

been shown previously, that the phosphorylated V_2 receptor peptide induced a conformational change in β-arrestins that appeared to correspond to their activation (Xiao et al. 2004)—although it is not clear whether it can indeed fully activate β-arrestins.

The two structures show remarkably similar overall changes (Fig. 7). Most notably, there is a rotation by about 20° of the C- vs. the N-terminal domain along the axis shown in the figure. This unanticipated rotation is, again, in contrast to the "clamshell" model, but it may also serve to expose interfaces in arrestins required for interactions with other downstream proteins. In addition, there are substantial rearrangements of the loops surrounding the polar core—in a manner similar to the changes predicted by the DEER measurements mentioned above (Fig. 7). The β-arrestin1 structure with the C-terminal V_2 receptor peptide also shows where the receptors' C-terminal tails bind, which is the "cup" of the N-terminal domain, i.e., the site where receptor binding had long been known to occur. However, the long postulated phosphate sensor, which critically involves Arg169 (Arg175 in visual arrestin), was not found to interact directly with the phosphopeptide as had been anticipated before (see above). However, binding of the phosphopeptide did disrupt the polar core, even without directly touching Arg169.

Thus, activation of arrestins appears to involve two types of structural changes: a rotation of the two halves, which may position its concave sides better towards active receptors, and a rearrangement of several central loops that may poise them for an interaction with the receptors. Whether this would allow binding of only one or perhaps two receptors remains to be elucidated. The many interactions between the phosphopeptide and β-arrestin1 presumably preclude a similar binding mode for a second receptor moiety (Kim et al. 2013)—but they do not rule out a more loosely attached second receptor, as suggested by the high photobleaching experiments on rhodopsin (see above). A more complete picture of these issues will require the structure of an entire receptor/β-arrestin1 complex.

6 Outlook

Arrestins have taken center stage in receptor research for more than two decades. During this time, they have been assigned an increasing number of functions and have become scaffold proteins that act as organizers of multiple signaling mechanisms, which they orchestrate in a temporally and spatially regulated manner. From a relatively simple shutoff mechanism, that was initially described for arrestin in the visual system (Wilden et al. 1986) and then for β-arrestins as general inhibitors of receptor signaling (Lohse et al. 1990b, 1992), the β-arrestins have emerged as control points for receptor internalization and trafficking, but also as triggers for multiple downstream signaling pathways. Even though visual arrestin can apparently also bind to some of these downstream signaling proteins (Song et al. 2006), most experiments assign these functions specifically to β-arrestins, and it remains to

be seen whether nonclassical, arrestin-mediated signaling plays a major role in the visual system.

While the basic mechanisms of the interactions between arrestins and receptors seem to be solved, major questions still wait for answers that must come from structures of the complexes: what is the nature of the conformational change that receptors induce in arrestins and vice versa, and how long does it take? Do receptors adopt distinct conformations in docking to arrestins vs. G proteins? Can receptors adopt multiple active conformations that allow them to distinguish between their different downstream partners, including the (β)-arrestins? What is the stoichiometry of receptor/arrestin complexes, and if two receptors can bind, what are the differences between the two interactions?

While these are at present mostly fundamental questions for the experimentalists, other issues may soon be transferred into real life. This includes attempts to engineer arrestins with designed functions (Gurevich et al. 2011; Gimenez et al. 2012) as well as the development of more selective, biased ligands. The potential of selective or biased activation that comes from the observation of differential signaling to G proteins and via β-arrestins is hoped to result in new types of therapeutics (Rajagopal et al. 2010; Ibrahim and Kurose 2012). Recently, β-arrestin-biased ligands have shown therapeutic potential in animal models of disease. For example, β-arrestin-biased angiotensin II AT_1 receptor stimulation has been reported to promote cell survival during acute cardiac injury (Kim et al. 2012a), and a novel prostaglandin EP_4 receptor-derived peptide thought to act via allosteric mechanisms has been reported to restore renal function in models of acute renal failure (Leduc et al. 2013). And finally, the first biased ligand, the β-arrestin-biased angiotensin II AT_1 receptor agonist TRV027, has recently completed the first phase I study in humans of such a compound (Soergel et al. 2013). The coming years will tell us about the therapeutic perspectives that these new classes of drugs may offer.

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Quantifying Biased β-Arrestin Signaling

Terry Kenakin

Contents

Abstract It is now established that agonists do not uniformly activate pleiotropic signaling mechanisms initiated by receptors but rather can bias signals according to the unique receptor conformations they stabilize. One of the important emerging signaling systems where this can occur is through β-arrestin. This chapter discusses biased signaling where emphasis or de-emphasis of β-arrestin signaling is postulated (or been shown) to be beneficial. The chapter specifically focuses on methods to quantify biased effects; these methods furnish scales that can be used in the process of optimizing biased agonism (and antagonism) for therapeutic benefit. Specifically, methods to derive $\Delta\Delta\text{Log}(\tau/K_A)$ or $\Delta\Delta\text{Log}(\text{Relative Activity})$ values are described to do this.

Keywords Arrestin • GPCR • Biased agonism • Cell signaling • Mathematical modeling

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1 Introduction

The canonical view of pharmacologic agonism considers seven-transmembrane receptors (7TMRs) to be rheostat-like switches providing a uniform signal (stimulus) to the cell of varying strength that is dependent upon the efficacy of the agonist. Within this model, agonists promote the stabilization of a single receptor active state; thus efficacy has a varying quantity but uniform quality. A considerable body of data published over the past 20 years clearly refutes this simple view and it is now accepted that agonists can produce varying ***qualities*** of stimulus to receptors as well (for reviews, see Kenakin and Miller 2010; Perez and Karnick 2005; Mailman 2007; Leach et al. 2007). The first formal model to describe this effect proposed the formation of ligand-specific receptor active states (Kenakin and Morgan, 1989; Kenakin 1995), although it should be noted that published descriptions of agonist profiles that did not agree with simple predictions of single-active state agonist models had been published some years earlier (i.e., Roth and Chuang 1987). The name first given to this phenomenon was "stimulus trafficking" although the terms "biased agonism" and "functional selectivity" have since become the accepted terminology. For the purposes of this chapter, the term "biased signaling" will be used since it can be applied to both agonists and antagonists. This chapter will specifically focus on the methods available to quantify biased signaling effects for the purposes of optimization through medicinal chemistry.

2 β-Arrestin-Mediated Signaling

Molecules that create biased activation of receptors that are pleiotropically linked to multiple signaling pathways in a cell theoretically can produce different efficacy-based phenotypic pharmacologic profiles. This chapter will discuss the measurement of biased signaling from agonists of 7TMRs that cause receptors, amongst an array of signals, to associate with β-arrestin; the outcome of this activity ranges from truncation of other 7TM signals (i.e., G protein activation), to receptor internalization and intracellular β-arrestin-based cellular signaling.

Historically, initial data suggested that the interaction of receptors with β-arrestin mainly caused the termination of the G protein coupling; subsequent studies have indicated a rich array of responses emanating from the β-arrestin intracellular complex (Luttrell et al. 1999; DeWire et al. 2007; Zhan et al. 2011; Ibrahim and Kurose 2012) resulting from ERK1/2 signaling to the suppression of constitutive activity of receptors (i.e., μ-opioid receptors, Walwyn et al. 2007). Biased signaling also can be shown to be a factor in the fine-tuning of signals through heterologous desensitization. For example, prolonged stimulation of μ-opioid receptors in brain locus ceruleus neurons has been shown to produce heterologous desensitization of α_2-adrenoceptor responses; this effect is abolished in β-arrestin2 knockout mice. This indicates that β-arrestin2 signaling can regulate

postsynaptic responsiveness in neurotransmitter release; the corollary to this finding is that biased μ-opioid agonists would therefore have differential effects on neurotransmitter release as well (Dang et al. 2012). The signaling outcome of receptor/β-arrestin interaction is known to be highly dependent on the type of receptor with which the β-arrestin interacts (Pal et al. 2013).

One of the earliest established favorable therapeutic actions of β-arrestin activation is its interaction with the angiotensin 1 receptor in cardiovascular disease. The first reported selective angiotensin receptor agonist for β-arrestin activation is SII ([Sar^1, Ile^4, Ile^8]angiotensin II) (Holloway et al. 2002) and it is with this ligand that much of the work was done to elucidate the benefit of β-arrestin activation (e.g., antiapoptic signal initiation, chemotaxis, cell growth and proliferation and cardiac contractility—see Ahn et al. 2009; Aplin et al. 2007a, b; DeWire et al. 2008; Hunton et al. 2005; Rajagopal et al. 2006). In general, β-arrestin-dependent ERK1/2 activation is associated with decreased apoptosis and increased activation of survival signaling in cardiac tissue. Activation of β-arrestin signaling may be a therapeutically useful property for drugs, especially those aimed at the treatment of congestive heart failure. For example, carvedilol is used for the treatment of congestive heart failure and part of its value may be its activation of a survival signal in cardiac muscle through a β-arrestin–Src–EGF signaling pathway (Noma et al. 2007; Kim et al. 2008). Similar effects are seen with angiotensin-mediated activation of the β-arrestin signaling pathway in cardiac muscle. Thus, activation of β-arrestin through angiotensin receptor 1 stimulation with the biased angiotensin ligands SII-AngII and TRV120027 (Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH) leads to increased cardiomyocyte contractility and performance and decreased cardiac fibrosis (Violin et al. 2010; Rajagopal et al. 2006). Also, selective β-arrestin activation with TRV120023 shows cardioprotection and diminished cell death in a mouse model of ischemic reperfusion injury (Kim et al. 2012). Similarly, the biased β-arrestin angiotensin agonist SII has been shown to produce reduced size of myocardial infarction in rat ischemia-reperfusion injury (Hostrup et al. 2012).

In addition to the evidence showing that β-arrestin signaling is relevant to diseases involving angiotensin (Tilley 2011b; Godin and Ferguson 2012), especially heart failure (Noor et al. 2011) and cardiovascular disease (Tilley 2011a; Noor et al. 2011; Lymperopoulos 2012), there is now a considerable body of evidence to implicate β-arrestin signaling in a host of other diseases including diabetes (Feng et al. 2011) and central nervous system diseases involving serotonin (Bohn and Schmid 2010), adrenergic signaling (Patel et al. 2010; Shenoy 2011), and parathyroid hormones (Viladarga et al. 2012). While the bulk of studies involve β-arrestin-2, there are reports that β-arrestin-1 signaling also can lead to selective biased signaling profiles notably through the GLP-1 receptor for diabetes (Sonoda et al. 2008) and with δ-opioid receptor desensitization (Aquila et al. 2012). Table 1 shows a partial list of disease areas where biased interaction of receptors with β-arrestin has been proposed to produce a favorable therapeutic profile. However, it should be noted that a biased molecule alters the signaling characteristics of natural endogenous molecules and there is no guarantee that this will induce only a positive quality to signaling. Table 2 shows cases where a β-arrestin activating property has

Table 1 Proposed biased ligands as improved therapies

Proposed therapy	Target	Pharmacologic effect	References
Osteoporosis	PTH	β-Arrestin-2 controls bone formation	Gesty-Palmer et al. (2009), Bohinc and Gesty-Palmer (2011)
Diabetes	GLP-1	β-Arrestin-1 mediates GLP-1-induced insulin secretion, antiapoptosis	Dalle et al. (2011), Sonoda et al. (2008)
Endothelial cytoprotection	PAR1	Activated protein C produces β-arrestin-2-mediated protective effects through PAR1R	Mosnier et al. (2012)
Acute myocardial injury	AT(2)	Diminished cell death in ischemia due to β-arrestin-2-mediated MAPK and Akt signaling	Kim et al. (2012)
Cardiac reperfusion injury	AT(I)	β-Arrestin-mediated signaling reduces infarct size in myocardial ischemia	Hostrup et al. (2012)
Psychosis	Dopamine D(2) R	β-Arrestin-2-mediated signaling mediates antipsychotic action	Chen et al. (2012), Allen et al. (2011)
Apoptosis	β-Arrestin-2	β-Arrestin-2 inhibits cell apoptosis through ERK1/2, p38 MAPK, Akt signaling	Chen et al. (2012)
Cytoprotection	Activated protein C	β-Arrestin-mediated signaling promotes cytoprotection	Soh and Trejo (2011)
Hypertension	α_2-Adrenoceptor	Hypotension with less sedation	Schmid and Bohn (2009)
Schizophrenia	Dopamine D_2 receptor	Improved treatment of schizophrenia reduced hyperalgesia	Grady et al. (2003), Urban et al. (2007)
Neuropsychiatric/ neurodegenerative disorders	Histamine R		
Thyroid hormone deficiency	Thyroid-stimulating hormone receptor	Selective thyroid hormone synthesis	Vassart and Dumont (1992)
Congestive heart failure	Angiotensin receptors	Cardioprotection concomitant with angiotensin receptor blockade	Wei et al. (2003), Rajagopla et al. (2005, 2006), Aplin et al. (2009), Violin and Lekowitz (2007), Violin et al. (2010), Zhai et al. (2005)

(continued)

Table 1 (continued)

Proposed therapy	Target	Pharmacologic effect	References
Parkinsonism	Dopamine D_1 receptors	Dopamine D1 receptor internalization	Ryman-Rasmussen et al. (2007), Ji et al. (2006)
	5-Hydroxytryptamine		Ji et al. (2006)
Treatment of addiction	Receptors gastrin-releasing peptide/ Arg vasopressin receptors	Receptor blockade + ERK stimulation	MacKinnon et al. (2005)
Small cell lung cancer—treatment of psychosis and depression	5-HT receptors	5-HT receptor internalization	Willins et al. (1999)

been postulated to be negative. In practice, once a biased molecule is identified, all aspects of signaling must be explored to ensure that a favorable overall profile is observed therapeutically.

Two approaches usually are utilized to evaluate β-arrestin signalling bias in drug therapy. One involves cases where a β-arrestin–receptor interaction is identified as being especially beneficial or especially harmful to a defined therapy; genetic knockout animals can be very useful in this regard. For example, the activation of the nicotinic acid receptor (GPR109) lowers serum fatty acids with accompanying debilitating flushing; this secondary negative effect is not observed in β-arrestin null mice (Walters et al. 2009), suggesting that a biased agonist for GPR109 devoid of β-arrestin activating effects would be a better therapeutic approach. Similarly, it has been shown that the normal respiratory depression associated with opioid receptor agonist-mediated analgesia is diminished in β-arrestin knockout mice. These data indicate that a biased ligand that does not cause receptor association with β-arrestin would be a better therapy for pain (Raehal et al. 2005; Bohn et al. 1999; Xu et al. 2007a, b; Groer et al. 2007). In this regard, the μ-opioid-biased agonist TRV130 ([(3-methoxythiophen-2-yl)methyl]({2-[(9R)-9-(pyridine-2-yl)-6-oxaspiro[4.5]decan-9-yl]ethyl})amine has been shown to produce analgesia in mice with less gastrointestinal dysfunction and respiratory depression than morphine through selective G protein signaling (and less β-arrestin recruitment) (DeWire et al. 2013). Another instance where β-arrestin knockout mice have been instrumental in identifying a superior therapy is in the treatment of osteoporosis with parathyroid hormone receptor agonists. Specifically, it has been shown that parathyroid hormone does not build bone or increase the number of osteoclasts in β-arrestin-2 knockout mice, indicating that this signaling pathway is the therapeutically relevant one (Ferrari et al. 2005; Gesty-Palmer et al. 2006, 2009). In this case, the data support the idea that a PTH agonist with biased signalling toward β-arrestin would be an optimal therapy for this receptor.

In other instances there is no clear rationale for knowing whether the selective activation (or selective avoidance) of β-arrestin will be a useful therapeutic

Table 2 Proposed therapies where β-arrestin activation may be unfavorable

Proposed therapy	Target	Pharmacologic effect	References
Analgesia	Opioid R	β-Arrestin mediates opioid respiratory depression	Xu et al. (2007a, b), Groer et al. (2007)
Reduction of triglycerides	GPR109	β-Arrestin mediates vasodilation leading to flushing	Kammermann et al. (2011)
Cancer	ET1	β-Arrestin-1 promotes metastasis in epithelial ovarian cancer	Rosano et al. (2013)
Cancer	IGF-1R	Ab for IGF-1R internalizes IFG-1R to cause ERK signaling	Zheng et al. (2012)
Anxiety/ schizophrenia	CB2	β-Arrestin-2-mediated ERK signaling through CB2R leads to upregulation of 5-HT(2A)R	Franklin et al. (2012)
Alzheimer's disease	β-Arrestin-2	β-Arrestin-2 overexpression leads to increased amyloid- β peptide to increase amyloid pathology	Thathiah et al. (2013)
Neural damage	AT(2)	β-Arrestin-2 mediates cerebral signaling to cause neural damage	Zhang et al. (2012)
Cardiac fibrosis	β-Adrenoceptor	Metoprolol-induced cardiac fibrosis leads to cardiac dysfunction through β-arrestin-2 signaling	Nakaya et al. (2012)
Chronic myelogenous leukemia	β-Arrestin-2	β-Arrestin-2 shown to be essential for CML disease propagation	Fereshteh et al. (2012)
Thrombosis	β-Arrestin-1	Allbb3 signaling activates β-arrestin-1 promotion of thrombus formation	Schaff et al. (2012)
Motor/psychoactive effects	CB1	β-Arrestin-2 signaling mediates tolerance, motor suppression to CB2 agonism	Nguyen et al. (2012)
Prostate cancer	β2-Adrenoceptor	β-Arrestin-2-mediated signaling mediates cancer progression	Zhang et al. (2011)
Hyperaldosteronism	AT(1)	β-Arrestin-1 overespression promotes aldosterone after myocardial infarction	Lymperopoulos (2012)
Myeloid leukemia	β-Arrestin-2	β-Arrestin-2 essential for disease progression	Fereshteh et al. (2012)

property; in these cases, the initial screening process for drug discovery can be modified to identify the possible relevance of β-arrestin bias. In this strategy, biased molecules are identified for further study in animal models to determine possible unique therapeutic phenotypes. Specifically, the active molecules from a given screen utilizing one signaling pathway are retested in a β-arrestin functional assay to determine differences in activation of either pathway. This process identifies intrinsically different molecules that can be tested in complex animal models for the possible identification of unique therapeutic phenotypes. As a preface to the discussion of the quantification of signaling bias, it is important to discuss what is meant by this term.

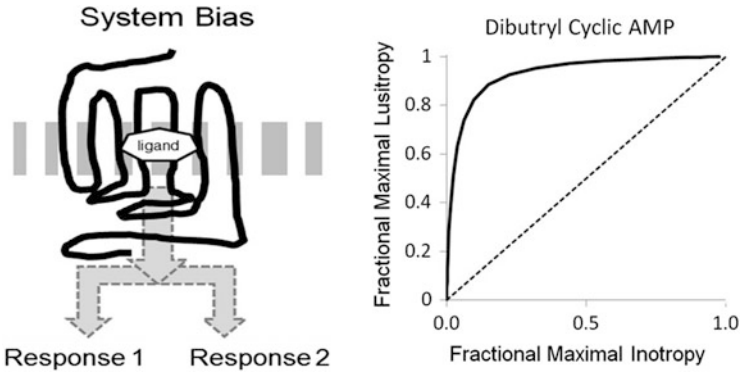

Fig. 1 Bias plot showing the relative effects of elevation of intracellular cyclic AMP levels in rat atria on inotropy (abscissae) and lusitropy (ordinates); the skewed relationship indicates a bias toward the process of lusitropy which is consistent with a mechanism whereby less cyclic AMP is required to induce positive lusitropy when compared to inotropy

3 Definitions of Signaling Bias

Pharmacologically, the term bias simply describes the phenomenon whereby activation of a receptor causes disproportionate activation of a given cellular signaling pathway linked to that receptor vs. the activation produced by another ligand. A generic method of expressing biased signaling is with a "bias plot"; specifically this is where the response produced by a given agonist in one signaling pathway is expressed as a function of the response produced by the same concentration of agonist in another pathway. There are three types of signaling bias: system, observation, and ligand bias; the first to be considered is *system bias*. As an illustration of this type of bias, it can be shown that dibutryl cyclic AMP produces positive inotropy and positive lusitropy in rat atria and that the lusitropic response is two- to threefold more sensitive than the inotropic response (Kenakin et al. 1991). A logical assumption in this case would be that the efficiency of physiological coupling of these responses differs, specifically that it requires less intracellular cyclic AMP to induce lusitropy than inotropy. Under these circumstances, a bias plot of these two responses shows a curved relationship, i.e., dibutryl cyclic AMP is biased toward selective lusitropy as opposed to inotropy (see Fig. 1). This is the result of an inherent property of the cell and not specifically the receptor stimulus. While in theory such bias might be exploited therapeutically (for instance, prenalterol, a weak β-adrenoceptor agonist produces selective lusitropy in rat atria) (Kenakin et al. 1991), the effect is cell type dependent and thus of very limited application for human therapy. Another type of bias involves the assays used to measure the signaling.

When two signaling pathways are visualized through separate assays, then the relative sensitivities of the assays can impart a bias on the relative response; this will be referred to as *observation bias*. For example, β-adrenoceptor activation of $G_{\alpha s}$ protein can be visualized by measuring intracellular levels of cyclic AMP

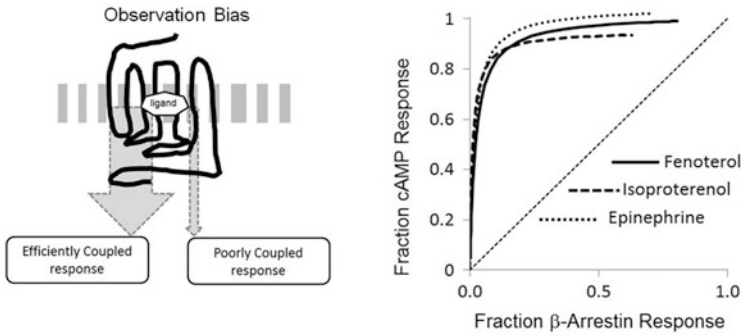

Fig. 2 Bias plot comparing the elevation of cyclic AMP as a function of β-arrestin receptor-association with β-adrenoceptor activation. Due to the fact that the cyclic AMP assay is much more sensitive than the β-arrestin assay, a bias is seen toward the cyclic AMP response. However, all three agonists produce the same bias indicating that this observation bias does not involve an agonist–receptor specific conformation. Plots re-calculated from data in Rajagopal et al. (2011)

pathways. Receptor interactions with β-arrestin can be monitored with enzyme complementation assays (Bassoni et al. 2012), bioluminescence resonance energy transfer (BRET) assays (Salahpour et al. 2012), and bimolecular fluorescence complementation (BiFC) assays (Kilpatrick and Holliday 2012). Figure 2 shows data where the association of the same β_1-adrenoceptors with β-arrestin can be observed with an enzyme cleavage-TANGO assay whereby β-adrenoceptor and β-arrestin association results in cleavage of a transcription factor which translocates to the nucleus to transcribe a stably expressing luciferase reporter gene (Rajagopal et al. 2011). The assay for β-arrestin association is 30-fold less sensitive than the assay for cyclic AMP leading to a clear bias in responses toward generation of cyclic AMP (see Fig. 2). It can be seen that the same bias is operative for the β-adrenoceptor agonists fenoterol, isoproterenol, and epinephrine, i.e., this is a general phenomenon common to all agonists for these two assays resulting from the difference in assay sensitivity. In general, observational bias will vary with assay conditions and types of assays and will be constant for all agonists; therefore it cannot be exploited for therapeutic use.

Both system-based and observation-based biased effects can be canceled by comparing agonists to each other in the same system. Thus, ratios of values (*vide infra*) are compared to a common agonist to unveil ligand bias. The therapeutically exploitable *ligand bias* is related to the chemical structure of the ligand and transcends system and observation bias. This property enables ligands to selectively activate defined signaling pathways in all cells and is due to the ability of the molecule to stabilize different conformations of the receptor—Fig. 3 (Kenakin and Morgan, 1989; Kenakin 1995). Thus, biased signaling occurs when the specific receptor conformations stabilized by the ligand selectively interact with signaling proteins in the cell; an example is shown in Fig. 4 where the two signaling proteins are G protein and β-arrestin. In this case the activation of β_1-adrenoceptor-mediated G protein and β-arrestin, by the agonists CGP 12177, albuterol, and clenbuterol, is

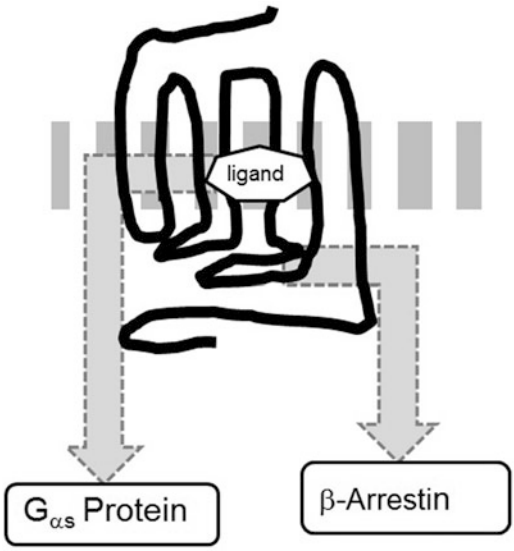

Fig. 3 True ligand bias emanates directly from the active receptor conformation stabilized by the agonist. Therefore, each ligand-specific conformation has varying efficiencies of interaction with subsequent signaling proteins such as G proteins and β-arrestin

used in a bias plot (ordinates = G protein activation; abscissae = β-arrestin activation; all values % of maximal effect to epinephrine). It can be seen that the responses are biased, but, unlike the uniform effects seem with observation bias (Fig. 2), there is a range of effects in that CGP 12177 is biased toward G protein signaling while both albuterol and clenbuterol are biased toward β-arrestin (clenbuterol > albuterol; see Casella et al. 2011).

The two settings for bias in practical drug therapy involve the emphasis of a given (presumably favorable) signaling pathway or the deletion of a given (presumably negative) signaling pathway. As molecules are optimized for biased activity, the relevance of any bias should become experimentally evident; a major tool in this endeavor is a practical scale to gauge the bias of molecules through quantification.

4 The Quantification of Biased Signaling

4.1 Transduction Coefficients

A model for agonism is required for the quantification of biased effects; in this regard the Black/Leff operational model (Black and Leff 1983) is extremely useful. Thus, agonism is described by the equation:

$$\text{Response} = \frac{[\text{A}]^n \tau^n E_\text{m}}{[\text{A}]^n \tau^n + ([\text{A}] + K_\text{A})^n}, \tag{1}$$

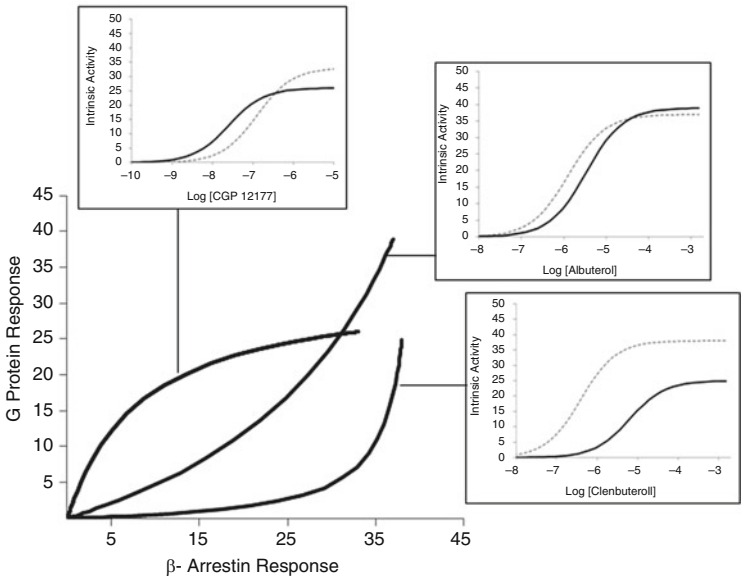

Fig. 4 Ligand-directed signaling bias: Activation of β_1-adrenoceptor by CGP 12177, albuterol, and clenbuterol; *solid line* represents interaction of the receptor with G protein, *dotted line* the interaction of the receptor with β-arrestin. The bias plot (% of epinephrine maximal response for each signaling pathway) shows how CGP 12177 is biased toward G protein signaling while both albuterol and clenbuterol are biased toward β-arrestin (clenbuterol > albuterol). Data drawn from data by Casella et al. (2011)

where [A] is the agonist concentration, E_m the maximal response capability of the system, n the slope of the concentration response curve, K_A the equilibrium dissocation constant of the agonist–receptor complex, and τ the efficacy of the agonist. To facillitate the statistical analysis of biased effects, it is useful to have agonism described by a single number. In addition, the scale must incorporate both the magnitude of the maximal response and also the potency (along the concentration axis of the concentraton–response curve) of the agonist. A theoretically sound index of agonism to yield a single number is a ratio of $\text{Log}(\tau/K_A)$ (referred to as a "transduction coefficient," Kenakin and Miller 2010; Kenakin et al. 2012; Kenakin and Christopoulos 2013). This index allows the quality of the agonist–receptor complex that is presented to the signaling protein to be characterized by τ and the allosterically modulated affinity of the agonist for the agonist–signaling protein complex to be described by K_A. The τ/K_A ratio thus characterizes the allosteric vector of agonist–receptor–signaling protein (Kenakin and Miller 2010). Therefore, the biased signaling associated with a receptor conformation formed by a ligand-dependent ternary complex of agonist, receptor, and signaling protein is quantified a single number ($\text{Log}(\tau/K_A)$) which is then compared to a common standard agonist (usually the natural endogenous agonist). This yields $\Delta\text{Log}(\tau/K_A)$ values which cancel system and observational biased effects and unveil true ligand-based biased

signaling. While the impact of the ternary complex is evident in bias (as the τ values), the possibility that agonist affinity may vary with signaling pathway may not be intuitively obvious.

There are two aspects of agonist affinity that may be operative in functional cells. The first is the impact of agonist efficacy on the makeup of the ensemble of receptor conformations present in a natural system. Proteins exist in numerous interconvertible states referred to as ensembles (Hilser and Freire 1997; Hilser et al. 1998; Fraunfelder et al. 1988, 1991; Onaran and Costa 1997; Onaran et al. 2002; Kenakin 2002) and if the agonist has differential affinities for these states (as would be predicted), the makeup of ensemble naturally will change with ligand binding (Kenakin and Onaran 2002). Reducing these numerous states to two for simplicity, the binding of a ligand to a two-state system can be represented as follows:

$$\mathrm{A} + \mathrm{R} \underset{}{\overset{K}{\rightleftharpoons}} \mathrm{AR} \underset{\varphi}{\overset{\gamma}{\rightleftharpoons}} \mathrm{AR}* \tag{2}$$

In this type of system, the functional affinity of the ligand is a mixture of binding reactions that can be represented as (Colquhoun 1985):

$$K_{\mathrm{A}} = \frac{K}{1 + \frac{\gamma}{\varphi}}. \tag{3}$$

Under these circumstances, the actual operational affinity of the agonist for the receptor [K_{A} in Eq. (3)] will depend on the amount of change in receptor conformation imparted to the system.

A second factor to be considered is the allosteric nature of 7TMR agonism. The affinity of agonists for receptors will be subject to the nature and amount of guest molecule co-binding to the receptor (Kenakin and Miller 2010; Christopoulos and Kenakin 2002; Christopoulos 2002). Under these circumstances, the binding of a ligand [A] in the presence of guest allosteric ligand [B] (with ligand equilibrium dissociation constants K_{A} and K_{B}) is given by

$$\frac{[\mathrm{ARB}]}{[\mathrm{R}_{\mathrm{tot}}]} = \frac{[\mathrm{A}]}{[\mathrm{A}](1 + \alpha[\mathrm{B}]/K_{\mathrm{B}}) + K_{\mathrm{A}}(1 + [\mathrm{B}]/K_{\mathrm{B}})}. \tag{4}$$

The presence of the allosteric guest [B] changes the affinity of the receptor for the ligand [A] by amount quantified by the factor α. From Eq. (4) the observed affinity of the receptor for ligand [A] in the presence of [B] is given by

$$K_{\mathrm{A(observed)}} = \frac{K_{\mathrm{A}}(1 + [\mathrm{B}]/K_{\mathrm{B}})}{(1 + \alpha[\mathrm{B}]/K_{\mathrm{B}})}. \tag{5}$$

As can be seen from Eq. (5), the operational affinity of the receptor for [A] is given by the nature of (α) and the concentration of the co-binding ligand B. Unless

the ligand has identical affinities for the receptor species ($\alpha \neq 1$), then $K_{A(observed)}$ will always be $\neq$ to K_A.

There is an abundance of experimental data to show that ligands binding to receptors directly affect the affinity of co-binding ligands. For example, the affinity of the NMDA receptor antagonist ifenprodil increases by a factor of ten in the presence of the co-binding ligand (NMDA) (Kew et al. 1996). Similarly binding studies with the allosteric ligand [^{3}H]dimethyl-W84 show that the affinity of the allosteric ligand gallamine for muscarinic M_2 receptors changes by a factor of 50 in the presence of the co-binding ligand *N*-methylscopolamine (Trankle et al. 1999). The same effects are seen when the co-binding ligand is a signaling molecule. For example, peptide fusion experiments with β_2-adrenoceptors in Sf9 membranes show a 27-fold increased affinity for isoproterenol upon the binding of nucleotide-free G_s heterotrimer protein to receptors (Rasmussen et al. 2011). Similarly, studies utilizing SCAM with κ-opioid receptors indicate changes in conformation in transmembrane domains 6 and 7 with binding of $G_{\alpha 16}$ and/or $G_{\alpha i2}$ G protein subunits; these result in an 18-fold change in the affinity of the ligand salvanorin (Yan et al. 2008). The effects of β-arrestin also have been described; specifically, the addition of β-arrestin and G_q protein to ghrelin receptor-containing lipid nanodiscs shows clear creation of different receptor conformations through double exponential fluorescent lifetime decay analysis (Mary et al. 2012). The fact that the affinity of ligands for receptor is conditional, that is, subject to the presence of co-binding species, suggests that biochemically derived estimates of affinity with binding experiments may have no relevance to the actual functional affinity of agonists in cells. For example, the binding affinity for ^{125}I-human calcitonin to human calcitonin receptors in HEK 293 cells is 16 pM ($pK_d = 10.77$ with 95 % confidence limits of 10.63–10.91, but the EC_{50} for calcium responses for human calcitonin is greater by a factor of 426 ($EC_{50} = 7.2$ nM, $pEC_{50} = 8.14 + 0.2$) (Watson et al. 2000). These differences occur presumably because the binding studies measure the total coupling of the receptor to all G proteins whereas the calcium response measures a particular G protein interaction. In view of the fact there is no a priori reason to suppose an affinity derived from binding studies is correct for functional activity, a functional affinity (K_A) is used that is obtained through fitting the equation to functional data. This yields a parameter referred to as a "transducer coefficient" defined as $\text{Log}(\tau/K_A)$ as the unique identifier of the ability of that agonist to activate that particular signaling pathway.

Ligand-specific signaling bias is made evident in the divergence of the curves shown in the bias plot (i.e., see Fig. 4); $\text{Log}(\tau/K_A)$ values can be used to quantify these divergences. In order to cancel system and observation bias, values must be calculated relative to a common standard molecule. For synthetic agonists, the natural endogenous agonist can be chosen since this will express the bias in terms of natural signaling. For the data shown in Fig. 4, the standard agonist used is epinephrine; $\text{Log}(\tau/K_A)$ values calculated from the predicted curves are then converted to ratios of τ/K_A values for epinephrine (for G protein vs. β-arrestin activation) in the form of $\Delta\text{Log}(\tau/K_A)$ values. Once $\Delta\text{Log}(\tau/K_A)$ are obtained for the two pathways as calculated with a common agonist, then a cross-pathway

Table 3 Biased signaling for chemokine activation of CCR5 receptors

Agonist	$\text{Log}(\tau/K_A)$	$\Delta\log(\tau/K_A)$[a]	$\Delta\Delta\text{Log}(\tau/K_A)$[b]	
IP1 production				
CCL3	7.75	0		
CCL4	8.01	0.26		
CCL5	8.27	0.52		
CCL3L1	8.48	0.73		
CCR5 internalization				BIAS[c]
CCL3	6.58	0	0	1
CCL4	8.2	1.62	1.36	23.1
CCL5	8.53	1.95	1.43	27
CCL3L1	8.82	2.24	1.51	32.4

[a]Vs. CCL3 $= \text{Log}(\tau/K_A)_{\text{CCIx}} - \text{Log}(\tau/K_A)_{\text{CCL3}}$
[b]Internalization vs. IP1 production : $\Delta\log(\tau/K_A)^{\text{Intl}} - \Delta\log(\tau/K_A)$
[c]$10^{\Delta\Delta Log(\tau/K_A)}$

comparison can be made through $\Delta\Delta\text{Log}(\tau/K_A)$ values (Kenakin et al. 2012); this is the logarithm of the bias. Therefore, the bias of the agonist is then defined as

$$\text{BIAS} = 10^{\Delta\Delta Log(\tau/K_A)}. \tag{6}$$

An example of the use of $\Delta\Delta\text{Log}(\tau/K_A)$ values to quantify bias for chemokines production of inositol phosphate vs. internalization of the CCR5 receptor is given in Table 3. It is worth discussing what these bias numbers mean. Specifically, they do not necessarily suggest that a given agonist preferentially activates a given pathway in a cell; this is determined by system and ligand bias. Rather, ligand bias denotes the preferential activation of a pathway relative to a standard agonist. Therefore, for the example shown in Table 3, while all agonists preferentially activate the IP1 pathway (over CCR5 internalization), CCL3L1 is 32.4 times more prone to activate internalization than is CCL3. The value in this scale is that it can be used within a given experimental system to optimize biased signaling through medicinal chemistry.

4.2 Relative Activity Values

Another scale that can be used to quantify biased signaling, described as "relative activity (RA)," has been proposed by Ehlert and coworkers (Ehlert 2005; Tran et al. 2009; Figueroa et al. 2009). RA values are defined as the maximal response to the agonist divided by the potency expressed as an EC_{50} value (concentration producing 50 % maximal response to the agonist). For concentration–response curves of standard slope (Hill coefficient $n = 1$), it can be shown that ΔLog (RA) values directly correspond to $\Delta\text{Log}(\tau/K_A)$ values. Specifically, from Black et al. (1985):

$$\mathrm{MAX} = \frac{\tau^n E_\mathrm{m}}{(1+\tau^n)}. \tag{7}$$

Similarly, the potency of agonists is defined as

$$\mathrm{EC}_{50} = \frac{K_\mathrm{A}}{\left((1+\tau^n)^{1/n} - 1\right)}. \tag{8}$$

It can be shown that relative activity equals

$$\mathrm{RA} = \frac{\tau^n \left((1+\tau^n)^{1/n} - 1\right) E_\mathrm{m}}{K_\mathrm{A}(1+\tau^n)}. \tag{9}$$

For $n = 1$, RA ratios for agonists 1 and 2 are $\tau_1 K_{\mathrm{A}2}/\tau_2 K_{\mathrm{A}1}$ (Griffin et al. 2007). This scale can be used to quantify the ligand-specific biased signaling shown in Fig. 4 for β_1-adrenoceptor agonist effects of G protein vs. β-arrestin activation. For these data, the bias [Eq. (6)] for CGP12277 is 15.7, albuterol = 1.56, and clenbuterol 0.17.

σ_{lig} and β_{lig} Values

A scale that is related to $\mathrm{Log}(\tau/K_\mathrm{A})$ has been proposed that utilizes only the efficacy (τ value) of the agonists to quantify bias; this scale employs a value termed σ_{lig} that is defined for agonists 1 and 2 as

$$\sigma_{\mathrm{lig}} = \mathrm{Log}(\tau_1/\tau_2). \tag{10}$$

While this scale uses the Black/Leff operational model to quantify agonism, it differs from the $\mathrm{Log}(\tau/K_\mathrm{A})$ scale in that the σ_{lig} scale is based on the assumption that the affinity of the agonist for the receptor is identical for both signaling pathways. Thus, it is proposed that this scale be employed using an independent estimate of affinity obtained from binding experiments. As discussed previously, there are theoretical and practical reasons why affinity estimates obtained for ligand-7TMR binding may not be applicable to functional estimates of agonist affinity; therefore it is logical to presume that there could be errors in the estimation of bias with this method for some ligands. The magnitude of the error will be dependent upon any difference in the affinity of the agonist for the receptor as it interacts with two different coupling proteins mediating different signaling pathways. For two agonists 1 and 2, the logarithm of the magnitude of that error in bias, when calculated with the σ_{lig} scale, is given as (Kenakin and Christopoulos 2013):

$$\Delta Log\ \mathrm{BIAS} = \mathrm{Log}\left[\frac{{K_{\text{A-1}}}^{\mathrm{path2}}}{{K_{\text{A-1}}}^{\mathrm{path1}}}\right] + \mathrm{Log}\left[\frac{{K_{\text{A-2}}}^{\mathrm{path1}}}{{K_{\text{A-2}}}^{\mathrm{path2}}}\right], \tag{11}$$

where $K_{A\text{-}1}$ and $K_{A\text{-}2}$ refer to the equilibrium dissociation constants of the agonists for the receptor when the agonists produce activation of two pathways path1 and path2 [denoted as superscripts in Eq. (11)]. Therefore, if the affinity of the agonist does not change as the agonist activates the two pathways, then there will be no difference between the σ_{lig} and $\mathrm{Log}(\tau/K_A)$ scales.

While the prediction of errors with the σ_{lig} scale depends upon projected differences in agonist affinity (as two separate signaling pathways are activated), there are data to show that there are an increasing number of systems where the σ_{lig} scale ***cannot*** be used to estimate bias. These are systems where τ values for each pathway activated by an agonist cannot be calculated with a single estimate of affinity. Table 4 shows a list of the agonists where a single value for agonist affinity mathematically will not yield values of τ for two signaling pathways. Thus, at least in these systems, it must be assumed that functional affinity changes with the activation of different signaling pathways. These data also show how a general assumption of uniform affinity is an erroneous assumption in the estimation of biased signaling.

5 Predicting Signaling Bias In Vivo

Different tissues in the body vary in their sensitivity to agonists according to their physiological needs; this occurs through difference in membrane receptor density, differences in the efficiency of coupling of receptors to cellular signaling pathways, or both. The robustness of the response to an agonist is mainly dependent upon its efficacy: high efficacy agonists will produce agonism in a greater variety of tissues than low efficacy agonists. The affinity of the agonist is not a factor in this effect since it only dictates agonist potency, i.e., at which concentrations agonism will occur when the tissue is sensitive enough to show it. For example, the low efficacy but high-affinity α-adrenoceptor agonist oxymetazoline produces full agonism in a sensitive tissue such as rat annoccygeus muscle, but a very weak response in a less sensitive tissue such as the vas deferens. In contrast, the low affinity but high efficacy agonist epinephrine produces powerful full agonism in both tissues (Kenakin 1984). This idea suggests that it is the efficacy of the agonist for the signaling pathway that dictates whether or not a given biased signal will be obtained in vivo, while the bias factor determines the relative concentrations at which it will be seen. Therefore, both the relative efficacy of the agonist as well as the bias must be considered in predicting biased signaling in vivo.

A useful representation of biased signaling can be made by plotting the relative maximal effect of agonists on two pathways as a function of the logarithm of their bias. Figure 5 shows that there are four general phenotypic activities that can be identified, which can be relevant to different applications of biased signaling. If the therapeutic aim of bias is to reduce a harmful effect of one of the pathways (i.e., β-arrestin effects such as those identified in Table 2), then useful compounds may reside in the upper quadrants A and B (Fig. 5). This representation shows the possibility that the affinity of the biased ligand may vary for the blockade of the β-arrestin effect in accordance with

Table 4 Significantly different pEC50 values for partial agonists activating two signaling pathways

Receptor	Agonist	EC_{50} (nM)	% Maximum	EC_{50} (nM)	Maximum	ΔEC_{50}	References
		Intracellular Ca^{2+} release		ERK1/2 phosphorylation			
5-HT_{2A}	5-Methoxy-DMT	60	83.2	8.23	89.8	7.29	Strachan et al. (2010)
	Lisuride	985	29.1	7.39	90.9	133.29	
	SCH-23390	329	25.7	21.2	51.3	15.52	
	MK212	817	72.5	133	71.2	6.14	
		[^{35}S]GTPγS binding		β-Arrestin-3 recruitment			
μ-Opioid	M6G	81	87	1,600	27	0.05	McPherson et al. (2010)
		CRE-Luciferase cyclic AMP		β-Arrestin-2 recruitment			
Histamine H4	VUF5228	39.8	47	398	17	0.10	Nijmeijer et al. (2012)
	VUF10185	126	82	1,000	17	0.13	
	VUF10306	398	83	1,000	60	0.40	
	VUF10778	12.6	79	63	27	0.20	
	VUF5222	6.3	92	39.8	37	0.16	
	VUF4704	100	73	1,000	50	0.10	
	VUF8328	3.2	77	63	73	0.05	
		G protein Activation		β-Arrestin activation			
β_1-adrenoceptors	Clenbuterol	6309	25	398	348	18.10	Casella et al. (2011)
		[^{35}S] GTPγS binding		β-Arrestin recruitment			
μ-Opioid receptors	Endomorphin-1	1.94	57	302	16	0.006	Nickolls et al. (2011)
	Endomorphin-2	3.2	55	288	16	0.011	
	Frakefamide	1.66	45	398	18	0.004	
	Morphine	1.78	59	524	6.7	0.003	
	Pfizer standard 1	0.46	53	19	25	0.024	

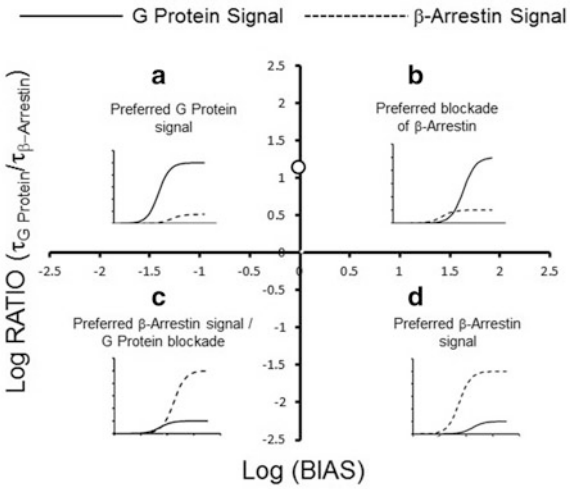

Fig. 5 Grid of bias ratios for agonists (values $\Delta\Delta\text{Log}(\tau/K_A)$) as the abscissae and Log of ratios of the efficacies of the agonist for each pathway as the ordinates. *Solid line* for G protein responses and *dotted line* for β-arrestin responses. Quadrant A contains molecules that primarily signal through G protein both in terms of maximal response and potency. The β-arrestin response of the natural agonist will be blocked. Quadrant B contains molecules that preferentially produce G protein response and the β-arrestin response to endogenous signaling will be blocked with a higher potency than G protein effects. Quadrant C molecules produce a preferential β-arrestin response with a preferential blockade of endogenous G protein signaling. Quadrant D molecules produce a preferential β-arrestin response with a low potency blockade of endogenous G protein signaling

the allosteric nature of receptors [Eq. (5)]; this effect has been observed as shown in the significantly different pEC_{50} values of partial agonists for two pathways shown in Table 4. On the other hand, if the therapeutically favorable biased effect is a selective augmentation of a pathway such as β-arrestin signaling, these would be found in lower quadrants C and D. An example of this type of representation is shown in Fig. 6, which shows the relative maximal effect of a series of β_1-adrenoceptor agonists as activators of G protein vs. β-arrestin expressed as a function of the bias of the same molecules for the same pathways. It can be seen, as an example, that while clenbuterol has a greater bias toward β-arrestin signaling (vs. epinephrine), fenoterol has a greater efficacy in producing this response (with a slightly lower bias than clenbuterol). Both the bias and the efficacy are relevant to the overall type of signaling that will be produced in vivo.

6 Future Directions

Over the past 20 years, the concept of signaling bias has been validated in a large number of receptor systems in vitro. In terms of a mechanism of biased signaling whereby the binding of ligands with differential affinity for an ensemble of receptor

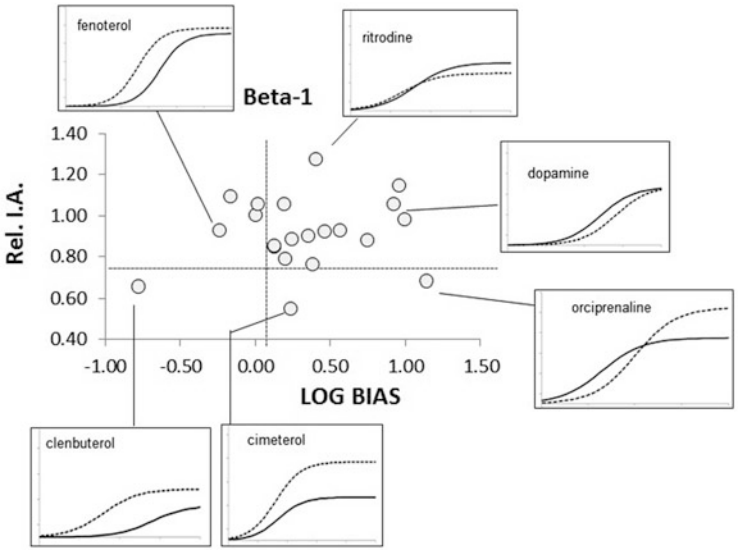

Fig. 6 Plot of β_1-adrenoceptor agonist activity for G protein activation (*solid line* concentration response curves) and β-arrestin activation (*dotted line* concentration response curves): abscissae are the Log Bias values for the agonists (from ΔΔLog(RA)) and ordinates are fraction of intrinsic activity values for the two responses. Data from Casella et al. (2011)

conformations produces the presentation of different ensembles of receptor to signaling proteins (Hilser and Freire 1997; Hilser et al. 1998; Fraunfelder et al. 1988, 1991; Onaran and Costa 1997; Onaran et al. 2002; Kenakin 2002), it would be expected that agonist bias would be a common occurrence. This is based on the prediction that different ligands would not be expected to have an identical array of micro-affinities for a large range of receptor conformations. This can be illustrated further with an equation showing the ratio of ligands in a reference conformational ensemble in the absence and presence of a ligand. This is given by (Kenakin 2013):

$$\frac{\rho_\infty}{\rho_0} = \frac{\sum_{i=1}^{n} \alpha_{i+1} L_{i+1} \left(1 + \sum_{i=1}^{n} L_{i+1}\right)}{\left(1 + \sum_{i=1}^{n} \alpha_{i+1} L_{i+1}\right) \sum_{i=1}^{n} L_{i+1}}, \tag{12}$$

where L_i is the allosteric constant determining the ratio of conformation i and a reference conformation 0 ($[R_i]/[R_0]$), and α is the relative affinity of the ligand for conformation i and 0; the ratio ρ_∞/ρ_0 is unity only when $\alpha\ i$ to n is equal to unity. What this means is the conformational ensemble will not change $(\rho_\infty/\rho_0) = 1$ only when the ligand has identical affintities for every conformational state in the ensemble. The corollary to this is that the distribution of conformational states in the ensemble will change if $\alpha \neq 1$ for any of the conformational states. Thus, it is quite probable that ligand binding will cause the presentation of a different receptor

ensemble of conformations to the cell and, unless the α values of two agonists are identical for each state, this ensemble will be different for each ligand. Under these circumstances, some form of bias would be expected of nearly every ligand. It is presently not clear to what extent this idea is supported by data since it requires a quantitative assessment of the number of ligands that have been tested to be biased and non-biased. However, one such study for 800 ligands of adenosine A(1) receptors indicated that G protein (over β-arrestin) signaling bias was found to be rare (one compound out of 800) (Langemeijer et al. 2013). It may be that the existence of ligand bias resides in certain regions of chemical space and that this pharmacologic property may be associated with different chemical scaffolds.

Intuitively it can be seen that for pleiotropically signaling receptors, ligand bias is a practical way of fine-tuning the output of a receptor. Thus, in so-called "redundant" systems such as chemokines, where a number of different chemokines are the natural ligands for the same receptor, it can be seen that different chemokines involved may induce different stimulus outputs from the same receptor (Zidar 2011). For example, the chemokines CCL19 and CCL21 are both natural agonists for the chemokine CCR7 receptor and both promote G protein signaling, but only one (CCL19) promotes agonist-induced receptor phosphorylation and recruitment of β-arrestin (Kohout et al. 2004). Similarly, it has been shown that the activation of the CCR2 receptor by agonists produces β-arrestin response of varying stability; while CCL8 and CCL13 produce stable CCR2–β-arrestin interaction, CCL7 produces a transient complex with a half-life of less than 15 min (Berchiche et al. 2011). These ligands also stabilize different conformations of the CCR2 homodimer (Berchiche et al. 2011). The same type of fine-tuning may be involved in the production of receptor isoforms. For example, GPR120 has long and short splice variants and it has been shown that agonist stimulation causes β-arrestin2–receptor association and receptor internalization for both splice variants but that the long splice variant selectively loses the ability to functionally produce G protein-dependent calcium and dynamic mass distribution effects. Thus, the short splice variant has been shown to be a naturally biased receptor (Watson et al. 2012).

In terms of the value of biased molecules in the therapeutic in vivo setting, the overall effect of a biased ligand in vivo is comprised of more than just biased direct signaling effects; two other effects are important, namely, the interference with endogenous signaling through the receptor occupancy of biased ligands and the possibility that phosphorylation-based barcoding of receptors may be different with different ligands. In some cases the blockade of endogenous signaling may be the more important therapeutic outcome of a biased ligand, as in the case of biased antagonists. These are molecules that bind to the receptor to preclude the activation of the receptor by the endogenous agonist and then impart an added cellular signal through a selective biased efficacy. Thus, the angiotensin antagonists SII (Wei et al. 2003), TRV120023 (Kim et al. 2012), and TRV120027 (Violin et al. 2010) bind to angiotensin receptors to block the debilitating effects of angiotensin in congestive heart failure (potent vasoconstriction) but also produce cell protecting β-arrestin-based cellular signaling through a biased β-arrestin efficacy. Specifically, studies in rats where blockade of endogenous angiotensin with the conventional

angiotensin receptor antagonists losartan or telmisartan leads to reduced mean arterial pressure and a decrease in cardiac performance. In contrast, the biased ligand TRV120027 actually increases cardiac performance and preserves cardiac stroke volume (Violin et al. 2010). This type of effect also has been observed in canine heart failure models where cardiac unloading actions with preserved renal function have been seen with TRV120027 (Boerrigter et al. 2011, 2012). Similarly, the biased histamine H4 receptor antagonist JNJ7777120 blocks agonist-induced G protein signaling of the receptor but actually promotes receptor interaction with β-arrestin (Rosethorne and Charlton 2011). Another case where the blockade of a natural signaling system with a biased ligand yields a favorable profile is with the dopamine D_2 receptor β-arrestin-biased agonist UNC9975 (7-(4-(4-(2,3-dichlorophenyl)-1,4-diazepan-1-yl)butoxy)-3,4-dihydro-1,8-naphthyridin-2(1H)-one). This partial agonist blocks the G_i protein-mediated cyclic AMP decrease by dopamine but concomitantly signals through β-arrestin agonism. This profile leads to antipsychotic activity in inbred C57B/6 mice in vivo ***without*** induction of debilitating motor side effects (Allen et al. 2011).

Another important aspect of receptor signaling that is becoming evident is the coding of receptor behavior after agonist binding, i.e., desensitization with or without internalization, recycling to the cell surface vs. degradation of receptors, etc. Specifically, receptor phosphorylation has been shown to code for receptor function after ligand binding (Tobin 2008; Tobin et al. 2008). Phosphorylation of receptors also has been shown to be an integral part of receptor interactions with β-arrestin. For example, phosphorylation-dependent and phosphorylation-independent interactions of β-arrestin2 and glucagon-like peptide-1 receptors have been reported (Jorgensen et al. 2011). The study of receptor phosphorylation has been facilitated by technological advances in the study of protein phosphorylation (i.e., tryptic phosphopeptide maps, phosphor-specific antibodies, mass spectrometry, Kelly 2011; Butcher et al. 2011); this, in turn, has indicated the presence of agonist-selective direct phosphorylation of receptor states. For example DAMGO and etonitazene, agonists of μ-opioid receptors, stimulate the phosphorylation of Thr370 and Ser375 whereas, in contrast, morphine leads to phosphorylation of only Thr370 (Doll et al. 2011). These patterns of phosphorylation "barcode" (Nobles et al. 2011; Butcher et al. 2011; Liggett 2011) receptors for future reference within the cytosol. Similarly, real-time multisite hierarchical phosphorylation of μ-opioid receptors occupied by DAMGO, fentanyl, sufentanil, and etorphine has been shown to be agonist specific (Just et al. 2013). Similarly, analogues of somatostatin (namely, SOM230 and KE108) have been shown to be less effective at stimulating the somatostatin sst2A receptor phosphorylation than somatostatin (Kao et al. 2011).

In general, functionally selective ligand profiles in vivo involve the following phenomena:

1. May produce direct selective signaling
2. May change the sensitivity of the various organs involved, as well as the intrinsic efficacy of the ligand for each pathway

3. May block the signaling by endogenous signaling via certain pathways
4. May change the phosphorylation patterns of receptors through stabilization of selective receptor conformations

In terms of future directions with respect to β-arrestin-based biased signaling, studies reveal a rich texture in the behavior of receptor–β-arrestin complexes once biased effects are imposed on the receptor and these can lead to different cellular outcomes. Largely unexplored areas in this regard are the impact of receptor dimerization on β-arrestin effects (Schelshorn et al. 2012; Sanchez-Martin et al. 2013; Heinrich et al. 2012) and the impact of different conformations of β-arrestin in the cell (Coffa et al. 2011; DeFea 2011; Zimmerman et al. 2012; Sauliere et al. 2012).

7 Conclusions

The demonstration of active β-arrestin-mediated cellular signaling coupled with the production of selective directed coupling of receptors to signaling proteins by agonists has necessitated the study of signaling profiles of new synthetic ligands and the quantification of the resulting responses. Quantitative system-independent scales to do this are vital to the orderly exploitation of these phenomena for therapeutic gain, and in this regard, the $\Delta\Delta\text{Log}(\tau/K_A)$ and $\Delta\Delta\text{Log(RA)}$ scales are very useful. As biased ligands enter the clinic, evaluations will be possible to assess the predictability of biased signaling from in vitro test systems to in vivo therapeutic ones.

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The Physiological Roles of Arrestin-1 in Rod Photoreceptor Cells

Jeannie Chen

Contents

Abstract Arrestin-1 is the second most abundant protein in rod photoreceptors and is nearly equimolar to rhodopsin. Its well-recognized role is to "arrest" signaling from light-activated, phosphorylated rhodopsin, a prototypical G protein-coupled receptor. In doing so, arrestin-1 plays a key role in the rapid recovery of the light response. Arrestin-1 exists in a basal conformation that is stabilized by two independent sets of intramolecular interactions. The intramolecular constraints are disrupted by encountering (1) active conformation of the receptor (R*) and (2) receptor-attached phosphates. Requirement for these two events ensures its highly specific high-affinity binding to phosphorylated, light-activated rhodopsin (P-R*). In the dark-adapted state, the basal form is further organized into dimers and tetramers. Emerging data suggest pleiotropic roles of arrestin-1 beyond the functional range of rod cells. These include light-induced arrestin-1 translocation from the inner segment to the outer segment, a process that may be protective

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against cellular damage incurred by constitutive signaling. Its expanding list of binding partners also hints at additional, yet to be characterized functions. Uncovering these novel roles of arrestin-1 is a subject of future studies.

Keywords Arrestin-1 • Rhodopsin • Rod photoreceptors • Signal shutoff • Translocation

1 Introduction

G protein-coupled receptors (GPCRs) are heptahelical transmembrane proteins responsible for sensing a remarkably wide range of extracellular signals that include photons, lipids, amino acids, peptides, and proteins, as well as other classes of organic or inorganic molecules. GPCR-initiated downstream intracellular biochemical cascades are of differing response amplitudes and durations, depending on the physiological context. This, in turn, is shaped by the rate of amplification as well as the recovery, where deactivation of the receptor plays a key role. A multistep process controls the catalytic lifetime of the GPCR: multiple phosphorylations by G protein receptor kinases (GRKs) followed by arrestin binding. With respect to the light-activated GPCR signaling in the rod photoreceptor cell, the single photon response is rapid and reproducible. This is necessitated by the fact that the physiological range of rods is limited to dim illumination, where single photon absorptions ultimately encode our visual scene.

The rod phototransduction cascade has led the way in the study of GPCR signaling for several reasons. First, the concentration of signaling molecules in rods is several orders of magnitude higher than in other cells in the body (Hamm and Bownds 1986; Pugh and Lamb 1993, 2000). Second, these molecules are compartmentalized in the outer segment which can be biochemically isolated (Papermaster and Dreyer 1974; Raubach et al. 1974; Papermaster 1982). Third, large amounts of rod outer segment (ROS) material can be obtained from frog or cow eyes, which, like humans, are rod-dominant. Fourth, absorption of a single photon leads to a change in current at the plasma membrane that can be measured by suction electrode recordings of intact rods, allowing for single molecule analysis (Baylor et al. 1979). The shape of the single photon response provides information about underlying biochemical cascade. Using a combination of transgenic mouse lines with targeted mutations to the phototransduction cascade and suction electrode recordings, along with comparing light responses from various mutant rods with that of normal responses, has provided detailed information about the mechanisms that regulate the phototransduction cascade. In some instances, defective signaling can lead to cell death, and these mouse models provide a platform for understanding the mechanism of retinal degeneration.

2 Arrestin-1 Rhodopsin Interaction: Structure/Function

The visual pigment rhodopsin is a prototypical GPCR expressed by retinal rods for photon absorption. Light sensitivity is conferred by 11-cis retinal, a chromophore that is covalently linked to the K296 residue of the opsin protein (Wald et al. 1950; Bownds 1967). The presence of the chromophore stabilizes the basal conformation of the rhodopsin, such that spontaneous activation occurs only about once every 700 years (Baylor et al. 1980). Photon absorption causes a cis to trans conformational shift in the retinal, which in turn leads to structural changes in the protein moiety. The Meta II conformation (R*) is the catalytically active form of rhodopsin that promotes the exchange of GTP for GDP on the visual G protein transducin. After activating many transducin molecules, R* is phosphorylated at Ser and Thr residues that are clustered near the carboxyl terminus (Wilden and Kuhn 1982; Thompson and Findlay 1984) by G protein-coupled receptor kinase 1 (GRK1), also known as rhodopsin kinase (Kuhn and Wilden 1982). When these sites are removed, or when GRK1 is knocked out, the single photon response shows an increased amplitude followed by a steady plateau and deactivates stochastically following the time course of R* decay, a relatively slow process where all trans retinal is hydrolyzed and dissociates from the opsin protein (Chen et al. 1995, 1999a). These studies show that phosphorylation plays a role in limiting the response amplitude as well as extinguishing the catalytic activity of R*. It is known that heavily phosphorylated rhodopsin shows very low catalytic activity toward transducin activation in vitro, raising the question of arrestin's physiological role in the recovery of the light response (Miller et al. 1986; Wilden 1995). This question was addressed using the arrestin-1 knockout mouse (Fig. 1) (Xu et al. 1997). Responses to increasing flash strengths are compared between WT and ARR1−/− rods (Fig. 1a, b). The rising phase of the responses was similar, indicating the same initial amplification gain. The ARR1−/− responses recover partially until a plateau is reached (Fig. 1b) and then return to baseline follows a stochastic process. A comparison of the single photon response from these rods again showed a normal rising phase and amplitude and diverged from the WT response after an initial phase of recovery that was attributed to rhodopsin phosphorylation. In contrast, the response from a mutant rhodopsin, S334ter that lacks all phosphorylation sites, showed increased amplitude and no rapid recovery (Fig. 1c). Taken together, these studies show that phosphorylation initiates deactivation, limits the response amplitude, and decreases the catalytic activity of rhodopsin by more than half. Therefore, arrestin-1 binding is required to completely quench the response. In the absence of arrestin-1 binding, the stochastic process of R* decay becomes the rate-limiting step for rhodopsin deactivation.

Human and mouse rhodopsin contains six Ser and Thr sites within a stretch of ten amino acids at the carboxyl terminus, whereas bovine rhodopsin contains seven of these sites (Fig. 2). The function of this cluster was investigated using transgenic mouse rods that express rhodopsin molecules in which selected phosphorylation sites were replaced by Ala (Mendez et al. 2000). Single photon responses recorded from these rods reflect the activity of individual mutant rhodopsins. It was found

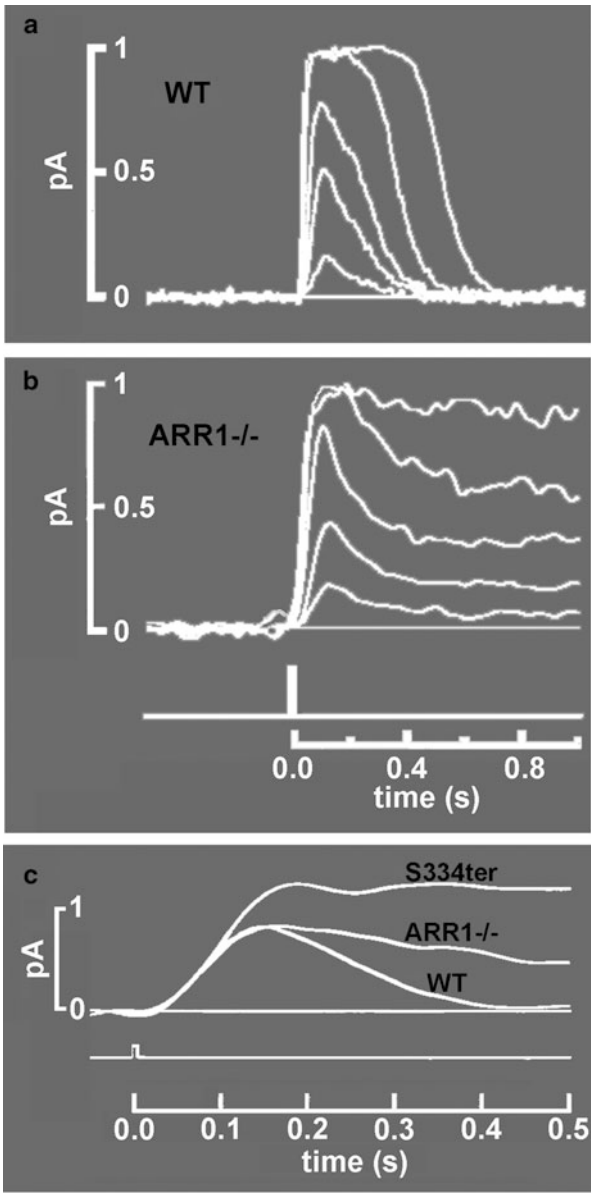

Fig. 1 Flash responses show prolonged recovery from ARR1−/− rods. The traces represent averaged normalized responses from WT (**a**) and ARR1−/− (**b**) rods to flashes of increasing strength. (**c**) A comparison of single photon responses from WT, ARR1−/− and S334ter rhodopsin that lack all phosphorylation sites. [Modified from Xu et al. (1997)]

Residue number	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348
Mouse	A	S	A	T	A	S	K	T	E	T	S	Q	V	A	P	A
Bovine	A	S	T	T	V	S	K	T	E	T	S	Q	V	A	P	A
Human	A	S	A	T	V	S	K	T	E	T	S	Q	V	A	P	A

Fig. 2 Comparison of rhodopsin's carboxyl terminal residues from indicated species. The Ser and Thr sites are colored *red*. The trafficking motif is colored *blue*

that when only one or two sites are available, the amplitude of the response was similar to that of normal, but the responses were step-like and recovery to baseline followed the decay of MII, as if arrestin-1 did not bind. When three Thr residues are available, deactivation was complete, albeit the responses were less reproducible (Doan et al. 2006). These observations are consistent with in vitro experiments that show the requirement of three phosphates for arrestin binding (Vishnivetskiy et al. 2007).

Arrestin-1 exists in a basal conformation that is stabilized by two distinct molecular "clasps" (Gurevich and Gurevich 2004; Gurevich et al. 2011). First is a network of five interacting charged residues that form the "polar core," and the second is the three-element interaction between the carboxyl terminal residues with β-strand I and α-helix I. In the basal state, arrestin exhibits low affinity binding to nonactivated, phosphorylated rhodopsin (R-P) or light-activated, non-phosphorylated rhodopsin (R*) through two distinct elements. High affinity binding occurs only when both elemens are engaged, i.e., when arrestin encounters R*-P (Gurevich and Benovic 1993). The mechanism of arrestin-1 activation is thought to involve initially charge–charge interaction of Lys14 and Lys15 with the multi-phosphorylated C-tail of rhodopsin and subsequently the disruption of arrestin's polar core by the negative charge of the phosphates. This conformational change exposes sites for high-affinity binding to R*-P. Because arrestin can be preactivated to bind R* by highly negatively charged molecules such as heparin sulfate (Palczewski et al. 1991; Gurevich et al. 1994), it is thought that the identity of the phosphorylated residues is not as important as their overall number. However, in vitro experiments using rhodopsin phosphorylated only at the three Ser sites show less binding to arrestin when compared to phosphorylation at three Thr sites (Brannock et al. 1999). In addition to amino acid identity, it is also possible that the relative position of the phosphates at the carboxyl terminus can affect the effectiveness of arrestin activation. The possible role of amino acid identity and position of phosphorylation at rhodopsin's carboxyl terminus in arrestin activation awaits functional analysis of suction electrode recordings of in intact transgenic mouse rods that express corresponding rhodopsin mutants.

2.1 The Concentration of Arrestin in Rods

For most studies rod outer segments (ROS) are typically obtained from dark-adapted retinas so that the ligand, i.e., photons, can be applied in a controlled manner to stimulate the phototransduction cascade. Using isolated outer segments

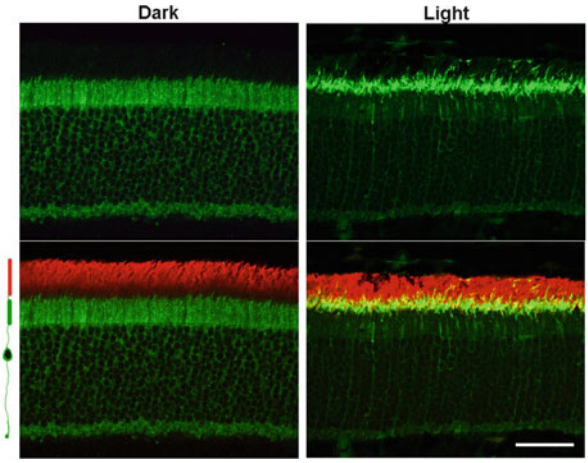

Fig. 3 Light induced arrestin-1 translocation. In the dark-adapted state, arrestin-1 (*green*, *upper left panel*) is localized in the inner segment, outer nuclear, and synaptic layers. Rhodopsin labeling (*red*) highlights the boundary between the outer segment and inner segment (*lower panels*). Light exposure causes arrestin-1 to move toward the outer segment compartment (*right panels*). Scale bar: 25 μm

from dark-adapted frog eyes, the concentration of arrestin-1 was found to be about one arrestin-1 molecule per ten rhodopsin molecules (Hamm and Bownds 1986). This value was also obtained for dark-adapted mouse eyes in more recent studies, which suggested a concentration of ~300 μM (Strissel et al. 2006; Song et al. 2011). Although the known function of arrestin-1 is in termination of the phototransduction cascade, the majority of arrestin-1 in the dark-adapted retina is located in the inner segment and synaptic compartments (Fig. 3). When these pools are considered, the total amount of arrestin-1 is close to 2.5 mM, only slightly less than the concentration of rhodopsin, which is 3 mM in ROS (Strissel et al. 2006; Hanson et al. 2007a). Arrestin-1 self-associates at 2.5 mM, forming dimers and tetramers (Imamoto et al. 2003; Hanson et al. 2007b). This phenomenon is observed in arrestins from different species, suggesting an evolutionary conserved function (Kim et al. 2011). Despite its tendency to oligomerize, the binding ratio of arrestin-1 to R*-P is one to one in the dim light regime (Hanson et al. 2007a). In the dark-adapted ROS, the concentration of active monomer ready to bind R*-P ranges from 15 to 50 μM depending on species (Kim et al. 2011). When nearly all rhodopsin is activated under bright light, one arrestin-1 may bind a second rhodopsin molecule, although with a lower affinity (Sommer et al. 2011).

2.2 Arrestin-1 Translocation

The tendency of arrestin-1 to be in the inner segment compartment in the dark-adapted cell (Fig. 3) may be due to the weak affinity binding of arrestin-1 monomers, dimers, and tetramers to microtubules (Nair et al. 2004; Hanson et al. 2006), which are abundant in the inner segment. Interestingly, light exposure causes arrestin-1 to move from the inner segment to the outer segment, coincident with

generation of its high affinity binding partner, R*-P (Calvert et al. 2006). This was first observed using immunocytochemistry of retinal sections obtained from dark-adapted and light-adapted mouse retinas (Broekhuyse et al. 1985) and later biochemically confirmed using tangential sectioning which allows for separation of different cellular compartments, followed by Western blots of this serially sectioned tissue (Strissel et al. 2006). Tangential sectioning is technically challenging, especially for the mouse retina due to its small size and highly curved shape. A particular strength of this technique is that it allows for quantitative measurements of protein levels in different rod compartments. Using this method, it was shown that arrestin-1 begins to move to ROS at a light intensity that generates at least 1,000 R*/s (Strissel et al. 2006), a light threshold at the upper limit of the rod's functional range. The rate of movement is equivalent to the calculated diffusion rate for a soluble protein with the halftime of ~5 min. The return to the inner segment following dark adaptation is substantially slower, with the halftime of 65 min (Strissel et al. 2006). The mechanism behind the light-induced translocation has been under intense investigation. Generation of the high-affinity arrestin binding target, R*-P, is required, inasmuch as no translocation was observed in the RPE65 knockout mouse retina where a defect in the visual cycle restricted the supply of the 11-cis retinal chromophore (Mendez et al. 2003). In these retinal sections arrestin-1 appeared diffusely located in all cellular compartments. However, the light threshold required to translocate arrestin and its super stoichiometric ratio to R* (>30:1) indicate that additional mechanisms are involved. One explanation could be the tendency of arrestin-1 to oligomerize in the inner segment. Another mechanism could be transducin signaling: although arrestin-1 translocation was observed qualitatively with immunofluorescence in retinas from transductin knockout mice (Strissel et al. 2006), its distribution in the dark and light-induced movement was abnormal and translocation was incomplete, based on the quantitative method of tangential sectioning followed by Western blot.

Does arrestin-1 translocate due to passive diffusion or active transport? Currently there are several lines of experimental evidence in support of the diffusion model. First, the arrestin-1-binding partners in different compartments in the dark (tubulin in the inner segment) and light (R*-P in the outer segment) are of sufficient abundance to act as "sinks" for arrestin-1 movement. Second, arrestin-1 movement proceeds in the absence of ATP (Nair et al. 2005). Third, diffusion measurement of GFP in rods, with the size comparable to arrestin-1, showed a diffusion rate similar to arrestin-1 movement (Calvert et al. 2006). Lastly, the amount of ATP required to move such a massive quantity of arrestin-1 is likely to be beyond the cellular capacity (Gurevich et al. 2011).

2.3 Functional Comparison of Arrestin-1 and Arrestin-4

The cone arrestin gene was identified by homology cloning following the cloning of rod arrestin-1 and the two beta-arrestins and was thus named arrestin-4 (Murakami et al. 1993; Craft et al. 1994). While arrestin-4 is expressed only in cones in the

retina, it was only realized much later that arrestin-1 is also expressed in cones (Zhu et al. 2005), at an amount estimated to be 50- to 200-fold more abundant than arrestin-4 (Chan et al. 2007; Nikonov et al. 2008). The ability of arrestin-1 and arrestin-4 to deactivate different visual pigments was compared using transgenic mice. When a short-wave cone opsin (S-opsin) was expressed in rods, it was demonstrated by suction electrode recordings that arrestin-1 deactivates S-opsin efficiently, following a similar time course as rhodopsin (Shi et al. 2007). Thus, arrestin-1 expressed in cones would be expected to participate in the recovery of the cone response. Indeed, cone light responses were largely normal in the arrestin-4 knockout mice and only became abnormally prolonged when both arrestin-1 and arrestin-4 were absent (Nikonov et al. 2008). In contrast, arrestin-4 deactivated rhodopsin poorly when it was expressed in rods lacking arrestin-1. Their light responses showed a greater extent of recovery when compared to the arrestin-1 knockout rods, indicating that it was able to reduce signaling from R*-P. Nevertheless the recovery was incomplete, as if high affinity binding did not occur (Chan et al. 2007). Therefore arrestin-1 and arrestin-4 are both capable of deactivating the cone pigment, but not functionally equivalent in deactivating rhodopsin.

3 Arrestin-1 in Health and Disease

3.1 Arrestin-1 Protects the Retina Against Light Damage

It has been long recognized that light exposure is an environmental factor that affect the health of photoreceptor cells. Light damage to the retina requires rhodopsin. When rhodopsin is knocked out, or when 11-cis retinal is absent, such as in the RPE65−/− mouse, the opsin molecules exist without the chromophore and light damage does not occur (Grimm et al. 2000). Light damage occurs through two distinct pathways. Photoexcitation of large numbers of rhodopsin molecules leads to generation of reactive oxygen species and lipid peroxidation in addition to stimulating the phototransduction cascade. Fortunately, the pigmented iris greatly limits the amount of light reaching the retina; therefore, bright light exposure normally does not harm the eye in a pigmented animal. However, when the pupil is dilated, or in the case of an albino animal lacking pigmentation, light exposure of >5,000 lux can cause photoreceptor cell death within hours (Noell et al. 1966; Gorn and Kuwabara 1967). This model of cell death triggered by massive photon absorption is dependent on activation of the transcriptional factors c-Fos and AP-1 (Hafezi et al. 1997; Wenzel et al. 2000). The other effect of light, which is constitutive stimulation of phototransduction, also appears to be deleterious. This was demonstrated in the arrestin-1 knockout (Chen et al. 1999b) as well as the GRK1−/− mouse (Chen et al. 1999a), where environmental light exposure of these pigmented mice lead to rod cell death followed by the death of cones. Because these mice were pigmented, the amount of photons reaching the retina was insufficient to

activate the oxidation pathway. Rather, the phototransduction pathway was amplified when the deactivation steps, i.e., rhodopsin phosphorylation and arrestin binding, were removed. This pathway of photoreceptor cell death was prevented when the arrestin-1 or GRK1 knockout mice were crossed into the transducin (GNAT1) knockout background, proving that constitutive signaling through the visual G protein was responsible (Hao et al. 2002). The two distinct light damage pathways activate different sets of transcripts during the initiating phase. Light induces activation of ATF-3 and ATF-4 transcriptional factors in the arrestin-1 knockout retina (Roca et al. 2004), as well as increase in global ubiquitination, suggesting involvement of the unfolded protein response pathway. In contrast, the transcription factors C/EBP ∂, c-fos and Egr-1 are activated in the Balb/c albino mouse model (Roca et al. 2004). Manipulating these two pathways may offer an avenue for enhancing photoreceptor cell survival.

3.2 *Functional Defect in Arrestin-1 Leads to Oguchi Disease in Humans*

Oguchi disease is a rare autosomal recessive form of congenital night blindness caused by mutations in the ARR1 gene (Fuchs et al. 1995) or GRK1 gene (Yamamoto et al. 1997). Visual acuity, visual field, and color vision are usually normal in these patients (Carr and Gouras 1965; Carr et al. 1966a). The time course of dark adaptation is extremely slow, while the adaptation of cones appears to proceed normally (Carr et al. 1966b), although in some instances cone function was also slightly affected (Cideciyan et al. 1998). This phenotype can be explained by the role of arrestin-1 and GRK1 in the recovery of the light response. In the absence of either, the effect of light is amplified and the rods saturate under low light. Because cones also express arrestin-4 and GRK7, the function of cones is less affected. Oguchi disease was considered to be stationary night blindness, meaning that visual defect persists only in rods. However, it was observed that some Oguchi patients gradually lost day time vision (Nakamachi et al. 1998), consistent with the observation in the mouse model that constitutive activation of the phototransduction pathway leads to rod cell death followed by cone death.

3.3 *Persistent Rhodopsin/Arrestin-1 Complex is Toxic to Rods*

Over 100 different mutations in the rod opsin gene have been found in humans diagnosed with retinitis pigmentosa (RP), the most common cause of inherited retinal degeneration which accounts for ~25 % of RP patients (Malanson and Lem 2009). Some of these mutations cause rhodopsin to mis-fold and trigger the

unfolded protein response (Mendes et al. 2005, 2010), others affect the C terminus of rhodopsin which contains a sequence motif, QVAPA, that serves as a trafficking signal to guide newly synthesized rhodopsin molecules in the inner segment to transport to the outer segment (Sung et al. 1993; Deretic et al. 1998; Hollingsworth and Gross 2012, Fig. 2). Mutations affecting the trafficking motif cause the mutant molecules to accumulate in the plasma membrane and in extracellular membrane vesicles prior to cell death (Sung et al. 1994; Concepcion et al. 2002; Concepcion and Chen 2010). Another class of mutations causes rhodopsin to be stuck in the "on" position. These include mutations affecting position K296, R135, and G90 (Robinson et al. 1992, 1994). K296 is the site of covalent attachment of 11-cis retinal through protonated Schiff base linkage (Wald et al. 1950; Bownds 1967). The visual pigment rhodopsin cannot form when K296 is mutated. Further, K296 forms a salt bridge with E113 when all-trans retinal is released from MII during the course of photon absorption (Nathans 1990; Cohen et al. 1992; Kim et al. 2004). Mutations that affect this salt bridge cause the opsin protein to show constitutive activity toward transducin activation when reconstituted in vitro. When the naturally occurring K296E mutant is expressed in transgenic mouse rods, it recapitulated the human disease by causing retinal degeneration (Li et al. 1995). However, K296E did not act as a source of molecular "dark light" to desensitize the rods. Instead, it was found persistently bound to arrestin-1 (Li et al. 1995). To see whether K296E–arrestin complex is the source of toxicity, the K296E transgene was crossed into the arrestin-1 knockout background. However, retinal degeneration was not prevented. Instead, the rods became desensitized and the outer segments were shortened, as would occur if the constitutive activity of K296E became unmasked in the absence of arrestin-1. To test the hypothesis that the mechanism of cell death is now G protein dependent, the mice were further crossed into the rod transducin (GNAT1) knockout background, whereupon the retinal morphology became much better preserved (Chen et al. 2006). This set of experiments supports the notion that persistent rhodopsin–arrestin-1 complex is toxic to rod cells. A similar mechanism of cell death was observed in Drosophila, where mutations leading to formation of stable rhodopsin–arrestin complex caused retinal degeneration (Alloway et al. 2000; Kiselev et al. 2000). Thus the toxicity of rhodopsin–arrestin complex appears to be conserved from fruit flies to mammals.

3.4 Functional Comparison Between Arrestin-1 and ß-Arrestins

Soon after the cDNA for arrestin-1 was isolated, homology cloning identified two other proteins with sequence similarity to arrestin-1 (Lohse et al. 1990; Attramadal et al. 1992; Sterne-Marr et al. 1993). Arrestin-2 and arrestin-3, also called ß-arrestins 1 and 2, are ubiquitously expressed and bind GPCRs that are phosphorylated by nonvisual GRKs in reactions analogous to arrestin-1 and visual pigments.

Over the past decade, new information has emerged regarding their roles in G protein-independent signal transduction. In particular, the ß-arrestins act as multifunctional scaffolds that interact with many protein partners and protein kinases and phosphatases, leading to changes in posttranslational modifications and distinct signaling outcomes (Shukla et al. 2011). Additionally, ß-arrestins contains binding sites for adaptor protein 2 (AP2) and clathrin, through which a plethora of signaling proteins is recruited (Schmid et al. 2006) during the process of clathrin-coated pit formation that was previously appreciated only for desensitization of surface receptors. Arrestin-1 does not contain a clathrin-binding domain, but does have an AP2 binding motif at its carboxyl terminus that confers weak binding in vitro (Laporte et al. 2000, 2002). This AP2-binding motif is absent in the naturally occurring arrestin-1 splice variant, p44 (Smith et al. 1994). Given that AP2 binding alone can recruit clathrin (Laporte et al. 2000), it is possible that arrestin-1 is able to recruit endocytic proteins via AP2. Indeed, recent evidence showed that K296E recruits AP2 and other key endocytic proteins, such as endophilin and clathrin, to the outer segment (Moaven et al. 2013). Interestingly, p44 rescued retinal degeneration and restored visual function to K296E mice (Moaven et al. 2013). These results implicate recruitment of endocytic proteins by the K296E/arrestin-1 complex in generating the cell death signal.

Additional non-rhodopsin-binding partners for arrestin-1 include tubulin; weak interaction between the abundant arrestin-1 and tubulin provides the basis for retention of arrestin-1 in the inner segment in the dark-adapted state (Nair et al. 2004; Hanson et al. 2006). More recently, MAP kinases JNK3 (Song et al. 2006), ERK2 (Hanson et al. 2007c; Coffa et al. 2011), Ca^{2+}-bound calmodulin (Wu et al. 2006), E3 ubiquitin ligase Mdm2 (Hanson et al. 2007c), parkin (Ahmed et al. 2011), NSF (Huang et al. 2010), and enolase (Smith et al. 2011) were identified as interacting partners with arrestin-1. The biological function of these interactions requires further investigation.

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Not Just Signal Shutoff: The Protective Role of Arrestin-1 in Rod Cells

Martha E. Sommer, Klaus Peter Hofmann, and Martin Heck

Contents

Abstract The retinal rod cell is an exquisitely sensitive single-photon detector that primarily functions in dim light (e.g., moonlight). However, rod cells must routinely survive light intensities more than a billion times greater (e.g., bright daylight). One serious challenge to rod cell survival in daylight is the massive amount of all-*trans*-retinal that is released by Meta II, the light-activated form of the photoreceptor rhodopsin. All-*trans*-retinal is toxic, and its condensation products have been implicated in disease. Our recent work has developed the concept that rod arrestin (arrestin-1), which terminates Meta II signaling, has an additional role in protecting rod cells from the consequences of bright light by limiting free all-*trans*-retinal. In this chapter we will elaborate upon the molecular mechanisms by which arrestin-1 serves as both a single-photon response quencher as well as an instrument of rod cell survival in bright light. This discussion will take place within

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the framework of three distinct functional modules of vision: signal transduction, the retinoid cycle, and protein translocation.

Keywords Arrestin • Rhodopsin • Opsin • All-*trans*-retinal • Retinoid cycle • Translocation • Stoichiometry

1 Introduction

1.1 The Visual System

The rod cell of the vertebrate retina is able to detect single photons by virtue of its cellular organization, the molecular structure of its photoreceptor rhodopsin, and the efficiency of the visual signal transduction module (Lamb and Pugh 2004; Hofmann et al. 2006, 2009). The rod outer segment (ROS) contains hundreds of flattened membranes (discs) that are densely packed with rhodopsin (Lamb and Pugh 2004). Rhodopsin is a G protein-coupled receptor (GPCR) composed of seven transmembrane helices (Fig. 1a) [see Hofmann et al. (2009)]. Visual signal transduction begins when the covalently linked inverse agonist of rhodopsin, 11-*cis*-retinal, absorbs a photon and isomerizes to all-*trans*-retinal (ATR). This event triggers a series of photo-intermediates that culminates in the active Metarhodopsin II (Meta II, Fig. 1a) (Matthews et al. 1963), which can couple to and activate the heterotrimeric G protein transducin. A single photon elicits a significant cellular response, because a single Meta II can activate hundreds of transducin molecules (Heck and Hofmann 2001), which go on to activate phosphodiesterase enzymes that rapidly hydrolyze intracellular cGMP (Hofmann et al. 2006). Meta II signaling is terminated by a multistep process. Rhodopsin kinase adds multiple phosphates to the C-terminal tail of the receptor (Wilden and Kuhn 1982), which allows the protein arrestin to bind and thereby block further interaction of Meta II with transducin (Wilden et al. 1986).

Meta II decays within minutes, when the Schiff base linking ATR to the protein is hydrolyzed, and Meta II releases a molecule of ATR resulting in the aporeceptor opsin. Arrestin-1 has been observed to modestly slow this process in vitro, but it cannot prevent it (Hofmann et al. 1992; Sommer et al. 2005). After ATR is released from the receptor, it is reduced by retinol dehydrogenase, and the resulting product all-*trans*-retinol diffuses to the nearby retinal pigment epithelium (RPE), where it is converted back to 11-*cis*-retinal by a complex enzymatic reaction sequence (McBee et al. 2001; Lamb and Pugh 2004; Wenzel et al. 2005). This retinoid cycle constitutes a functional module in which photolyzed ATR is re-isomerized to 11-*cis*-retinal in order to regenerate rhodopsin (Hofmann et al. 2006). In contrast to invertebrate rhodopsin, rhodopsin in vertebrates cannot be regenerated with a second photon absorption (Ritter et al. 2008). The complexity of the vertebrate retinoid cycle must confer some benefit by making photoreceptor regeneration

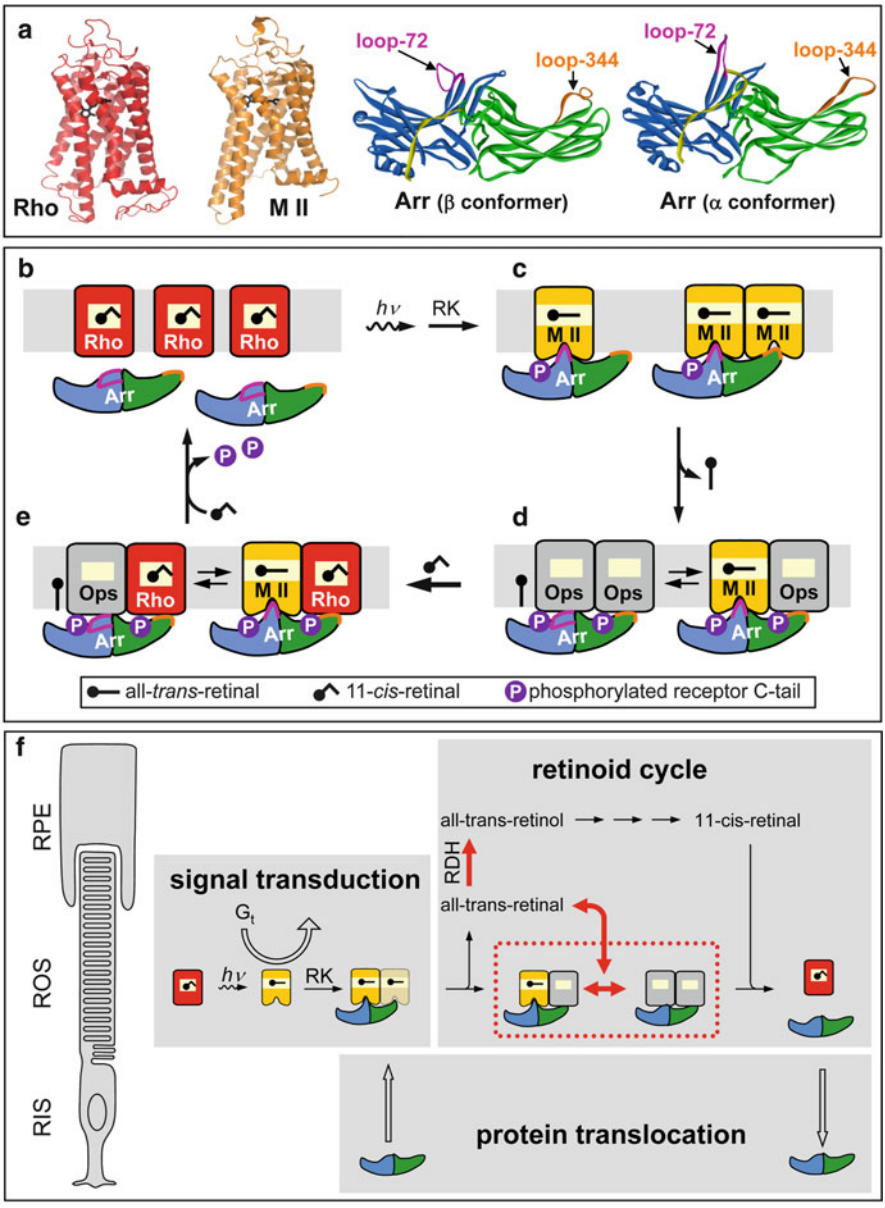

Fig. 1 Molecular, sub-modular and modular organization of arrestin and rhodopsin in the visual system. (**a**) Crystal structures of rhodopsin (Rho, Protein Data Bank accession 1U19), Meta II (MII, 3PXO) and arrestin (Arr, 1CF1). Two different crystallographic conformers of arrestin (α and β), which differ primarily in the flexible loops of the receptor-binding surface, are shown. For the arrestin models, the N-domain is colored *blue*, the C-domain is colored *green*, the C-tail is *yellow*, loop-72 is *magenta*, and loop-344 is *orange*. (**b**, **c**) Dark-state rhodopsin absorbs light to become Meta II and is phosphorylated by rhodopsin kinase (RK), thereby allowing arrestin to bind at variable stoichiometric ratios (see text for more details). Note that loop-72 (*magenta*) adopts a more extended conformation upon engagement of Meta II-P. (**d**) The arrestin/Meta II-P complex decays

independent of ambient light (Saari 2000) or is necessary to compensate for an unstable Meta II conformation that resulted from evolutionarily advantageous mutations in rhodopsin (Lamb 2009). Whatever the benefit may be, it comes at a high price, since large amounts of ATR are released at higher light levels. ATR is toxic, and its condensation products are linked to age-related macular degeneration (explained in more detail below).

The polarity of the rod cell (that is, having distinct inner and outer segments) is the basis of an additional functional module in vision: protein translocation. In the dark-adapted rod cell, most arrestin-1 is bound to microtubules within the inner segment (Nair et al. 2004). Conversely, most transducin is bound to the disc membranes in the outer segment. Exposure to light causes these two proteins to switch locations. It is still debated whether light-triggered protein translocation takes place solely by passive diffusion, or if energy-consuming molecular motors are involved as well (Nair et al. 2005; Orisme et al. 2010; Satoh et al. 2010). It is also debated whether protein translocation contributes to light adaption (Arshavsky and Burns 2012), serves to save energy, or simply helps the rod cell survive bright light (Gurevich et al. 2011).

1.2 Arrestin Structure and Function

The arrestin molecule consists of two roughly symmetrical clam-shaped lobes, termed the N- and C-domains, and each is composed of a β-sandwich (Fig. 1a) (Granzin et al. 1998; Hirsch et al. 1999). A long C-terminal tail (C-tail) interacts extensively with the N-domain and thereby holds arrestin in an inactive or basal

Fig. 1 (continued) into an equilibrium in which ATR can re-enter half of the arrestin-bound OpsP. ATR uptake is accompanied by an extension of loop-72. (**e**) 11-*cis*-retinal can enter the other OpsP to regenerate rhodopsin. Removal of ATR is required for complete regeneration which, together with dephosphorylation of OpsP, dissociates arrestin. (**f**) *Signal transduction module:* dark-state rhodopsin (*red*) absorbs light (hν) to form Meta II (*yellow*), which interacts with transducin (G_t) to initiate signaling in the rod cell. Meta II is phosphorylated by rhodopsin kinase (RK) and bound with high affinity by arrestin (*blue*, *green*) at either a one-to-one or one-to-two stoichiometry, thereby blocking signaling. *Retinoid cycle module*: arrestin-bound Meta II-P decays and releases all-*trans*-retinal, leaving OpsP (*gray*). All-*trans*-retinal can re-enter half of the arrestin-bound OpsP population to reform arrestin-bound Meta II-P, thus sequestering toxic all-*trans*-retinal within OpsP [*red dotted box*; for details see (**d**)]. Free all-*trans*-retinal is reduced to all-*trans*-retinol by retinol dehydrogenase (RDH). All-*trans*-retinol diffuses to the retinal pigment epithelium (***RPE***) where it is converted to 11-*cis*-retinal by a multistep enzymatic process. 11-*cis*-retinal is delivered back to the ROS were it recombines with OpsP to regenerate rhodopsin. *Protein translocation module*: arrestin diffuses from the rod inner segment (***RIS***) to the outer segment (***ROS***) upon exposure of the dark-adapted rod cell to light and is retained in the ***ROS*** by its interaction with Meta II-P and OpsP. Regeneration of rhodopsin dissociates arrestin and allows its return to the ***RIS*** during dark adaptation. The cellular and subcellular locations of the reactions are indicated by their placements with respect to the simplified illustration of the rod cell and the ***RPE*** cell on the *left*

conformation. Between the two lobes is a region called the polar core, a buried hydrogen-bond network composed of residues from both domains and the C-tail. Gurevich and colleagues have proposed a mechanism whereby initial engagement of receptor-attached phosphates by Lys14 and Lys15 in the arrestin N-domain destabilizes the local structure and results in release of the C-tail (Vishnivetskiy et al. 2000). C-tail displacement then triggers activating conformational rearrangements in arrestin that eventually promote tight binding to the active receptor (Schröder et al. 2002). Recently, our group gained insight into these activating conformational changes by solving the X-ray crystal structure of a C-terminally truncated splice variant of arrestin called p44 (Kim et al. 2013). Due to the lack of the regulatory C-tail, p44 exists in a preactivated state that is primed to bind the active receptor (Schröder et al. 2002). The activating conformational rearrangements observed in the crystal structure of p44 include a breaking of the polar core, an increase in interdomain flexibility, and a 21° rotation of the domains relative to one another (Kim et al. 2013). In addition, several key loops in the "central crest" region of arrestin, which have been observed experimentally to undergo conformational changes upon receptor binding (Hanson et al. 2006; Sommer et al. 2007; Kim et al. 2012; Vishnivetskiy et al. 2013), show significant displacements in p44 as compared to basal arrestin-1. Notably, strikingly similar conformational changes were observed in the crystal structure of nonvisual arrestin-2 (also called β-arrestin-1) in complex with a fully phosphorylated peptide derived from the V2 vasopressin receptor (Shukla et al. 2013). Together these crystal structures indicate that activation does not entail any large-scale change in the overall structure of arrestin, and changes in flexible loops are key in activating arrestin for receptor binding. In particular, we have identified two loops in either domain of arrestin, loop-72 (Gly68-Ser78, also called the finger loop) in the N-domain and loop-344 (Lue338-Ala348) in the C-domain (Fig. 1a), that play distinct roles in receptor binding (Sommer et al. 2012).

2 Arrestin Quenches Meta II Signaling

Arrestin-1 has long been known to play a central role in shutting off the signaling of active Meta II in the rod cell (Wilden et al. 1986; Xu et al. 1997). Since it was shown that arrestin-1 specifically binds and stabilizes phosphorylated Meta II (Meta II-P) at a one-to-one ratio (Schleicher et al. 1989), it was assumed that a single arrestin-1 couples to a single light-activated Meta II-P, as would be generated in a rod cell exposed to dim light. Indeed, arrestin-1 can functionally bind monomeric Meta II-P isolated in nanodiscs (Tsukamoto et al. 2010; Bayburt et al. 2011). However, the bilobed structure and size of arrestin-1 suggest that two receptors could be bound by a single arrestin-1, which would be advantageous when higher percentages of receptors are activated. Our recent work indicates that arrestin-1 can actually bind Meta II at different stoichiometric ratios in the native rod disc membrane, either one-to-one or one-to-two depending on the percentage of activated receptors and the average receptor phosphorylation level (Sommer et al. 2011, 2012). This variability

may arise from the different binding preferences of arrestin's two domains. According to this model, loop-72 (finger loop) within the N-domain specifically engages the active receptor (Sommer et al. 2012) and phosphate sensors within the N-domain bind the phosphorylated receptor C terminus (Fig. 1c). In contrast, loop-344 within the C-domain of arrestin-1 is less specific and can engage either the membrane surface (in the case of one-to-one binding) or a neighboring Meta II (in the case of one-to-two binding) (Fig. 1c). In the case of one-to-two binding, phosphorylation of the second receptor molecule that is engaged by the C-domain is not required (Sommer et al. 2012). This adaptability in binding mode may allow arrestin-1 to be an efficient signal quencher at different light intensities. Arrestin-1 binds monomeric Meta II-P at the low light levels in which the rod cell operates. At higher light intensities, arrestin-1 binding to Meta II-P can also inactivate a neighboring Meta II, even before this second receptor has been phosphorylated, thereby enhancing the signal-quenching efficacy of arrestin-1 and saving the energy required for receptor phosphorylation.

3 Arrestin Protects the Rod Cell from the Consequences of Bright Light

Soon after rod arrestin-knockout mice were created (Xu et al. 1997), it was reported that these mice suffer light-dependent retinal degeneration (Chen et al. 1999). Given the known role of arrestin-1, it was assumed that rod cell death occurred due to lack of signal shutoff. Indeed, knocking-out transducin expression protects arrestin-knockout mice from dim light-induced damage, yet unexpectedly does not protect from bright light-induced damage (Hao et al. 2002). This result suggests that retinal damage in bright light is not solely due to excessive signaling. Furthermore, bright light-induced damage is accompanied by induction of the proapoptotic transcription factor AP-1 (Hao et al. 2002; Reme 2005), the expression of which in human RPE cells has been shown to be upregulated by oxidative stress (Kalariya et al. 2008; Chaum et al. 2009). Based on these observations, we hypothesized that arrestin-1 protects the rod cell from toxic levels of ATR (see below). In support of this idea, we previously observed that arrestin-1 interacts with the products of Meta II-P decay, namely, phosphorylated opsin (OpsP) and ATR (Hofmann et al. 1992; Sommer et al. 2005). In the following sections, we discuss the physiological necessity for the limitation of free ATR and our findings regarding how arrestin-1 may accomplish this task.

3.1 Bright Light Generates Toxic Levels of ATR in the Rod Cell

Although necessary for vision, the high concentration of retinal in the rod cell presents a serious challenge to cell survival when light exposure induces the release

of ATR. The toxicity of ATR is apparent in the severe light-induced retinal degeneration suffered by mice that are unable to clear ATR after light exposure because they lack both retinol dehydrogenase (RDH) and the ABCA4 transporter (*Abca4*$^{-/-}$ *Rdh8*$^{-/-}$) (Maeda et al. 2008). RDH reduces ATR to all-*trans*-retinol (Rattner et al. 2000), and the ABCA4 transporter removes ATR from the disc interior (Weng et al. 1999). ATR is in itself toxic (Rozanowska and Sarna 2005; Wielgus et al. 2010) due to its reactive aldehyde component (Sparrow et al. 2010), consistent with the fact that drugs containing primary amines react with retinaldehyde to protect *Abca4*$^{-/-}$ *Rdh8*$^{-/-}$ mice from light-induced retinal damage (Maeda et al. 2011). ATR also photosensitizes cells to ultraviolet or blue light (Harper and Gaillard 2001; Rozanowska and Sarna 2005). In addition to its own toxicity, free ATR generates reactive oxygen species in the rod cell via a cascade of signaling events that stimulates the enzyme NADPH-oxidase (Chen et al. 2012), and cell death occurs from mitochondrial poisoning and caspase activation (Maeda et al. 2009). Furthermore, two molecules of ATR can sequentially react with phosphatidylethanolamine to form a pyridinium bisretinoid, so-called A2E, which collects over time in lipofuscin granules found in the RPE and is correlated with age-related macular degeneration (Sparrow et al. 2010).

In the situation of constant illumination, ATR is continuously generated by the photoactivation and decay of rhodopsin. At the same time, ATR is removed as RDH reduces it to nontoxic all-*trans*-retinol, which in turn diffuses to the RPE to be enzymatically converted back to 11-*cis*-retinal (Lamb and Pugh 2004). Constant illumination thus creates a steady state within the rod disc, meaning that the relative concentrations of rhodopsin, metarhodopsin, and opsin reach a constant level once the rate of photon absorption (i.e., bleaching) equals that of rhodopsin regeneration (Wenzel et al. 2005). This continuous cycle of photoactivation, decay, and regeneration results in a persistent population of ATR, which has been estimated at ~12–30 %[1] of the total amount of rhodopsin in mice kept under normal fluorescent laboratory lighting (Saari et al. 1998; Lee et al. 2010). With an estimated rhodopsin concentration in the mouse retina of ~3–5 mM (Lamb and Pugh 2004; Nickell et al. 2007), total ATR concentration (i.e., free and opsin-bound) would range between 360 μM and 1.5 mM in mice under normal room lighting. If only 10 % of this ATR were free, its steady-state levels would be 40–150 μM. Considering that as little as 100 μM ATR is toxic to cells in culture (Rozanowska and Sarna 2005), and only 25 μM ATR induces oxidative stress in RPE cells (Wielgus et al. 2010), even moderate indoor lighting presents a real risk to the murine retina.

In humans, the level of bleached rhodopsin is lower under a given amount of light as compared to mice, because rhodopsin is regenerated faster in the human retina (Lamb and Pugh 2004). Whereas normal room lighting (~100–200 cd/m^2) is sufficient to bleach half of rhodopsin in the mouse retina (Wenzel et al. 2005; Lee et al. 2010), bright daylight (~25,000 cd/m^2) is required to bleach half of rhodopsin

[1] The difference might be strain-related (Lamb and Pugh 2004), as the first study utilized one inbred albino strain and the second various pigmented strains.

in humans (Alpern 1971; Rushton and Powell 1972; Kaiser and Boynton 1996). This difference reflects the difference between the nocturnal mouse and the diurnal human. Although steady-state levels of ATR in human subjects have not been reported, it can be safely assumed that bright daylight generates potentially toxic levels of ATR in the human retina.

3.2 *Arrestin Stimulates Uptake of ATR by Phosphorylated Opsin*

Twenty years ago our group first reported that ATR stimulates arrestin-1 binding to phosphorylated opsin (OpsP) (Hofmann et al. 1992) and more recently we have determined the mechanism underlying this phenomenon. In the presence of arrestin-1, ATR can enter the binding pocket of OpsP and reform the retinal Schiff base, thereby reforming Meta II-P from OpsP and exogenous ATR (Sommer et al. 2012). Curiously, only half of OpsP receptors in the native rod disc membrane are able to take up ATR in the presence of arrestin-1. This asymmetry of ligand binding is explained by a binding model in which each domain of arrestin-1 functionally engages its own OpsP molecule (Fig. 1d). Loop-72 (finger loop) in the N-domain stabilizes the active conformation of opsin (Ops*) necessary for ATR uptake, whereas loop-344 in the C-domain engages the receptor but does not stimulate ATR uptake.

Considering the dangerous levels of free ATR potentially generated in the rod cell by daylight, arrestin-dependent uptake of free ATR by OpsP represents a valuable protective mechanism that would complement rod cell survival in bright light. Notably, the apparent K_D of ATR for arrestin-bound OpsP (3–5 μM) (Sommer et al. 2012) would be sufficient to reduce free-ATR concentrations to well below those that have been reported to be toxic (Rozanowska and Sarna 2005). Obviously, this proposition depends on the existence of arrestin-bound phosphorylated opsin in vivo. So far, available evidence suggests that highly phosphorylated opsin accumulates in the retina following light exposure (Kennedy et al. 2001; Shi et al. 2005; Lee et al. 2010). In the case of continuous illumination, Lee at al. found that 80 % of receptors were phosphorylated, even though only 55 % of rhodopsin was bleached in steady state (Lee et al. 2010), implying that a significant fraction of dark-state rhodopsin was phosphorylated under these conditions. Furthermore, most receptors incorporate multiple phosphates over time, because dephosphorylation of regenerated rhodopsin was slower than the rate at which a given rhodopsin molecule absorbed another photon (Lee et al. 2010). This finding is especially significant, considering that arrestin's affinity for OpsP scales with the number of phosphates per receptor (Gibson et al. 2000; Vishnivetskiy et al. 2007). These observations also hint at why rhodopsin contains so many phosphorylation sites (seven in bovine, six in mouse and human; see Chap. 4) when only three phosphates are required for high-affinity arrestin-1 binding to light-activated Meta II (Mendez et al. 2000; Vishnivetskiy et al. 2007). The additional phosphorylation

sites might be utilized for arrestin-1 binding to OpsP and arrestin-dependent uptake of ATR. Since arrestin-induced uptake of a single molecule of ATR requires two phosphorylated opsin molecules (Sommer et al. 2012), it can be envisioned that overall receptor phosphorylation level in the rod cell, which increases with relative light level (Lee et al. 2010), switches the rod cell from single photo-detecting to survival mode.

3.3 *Arrestin Imposes Asymmetric Ligand Binding Within an Opsin Dimer*

Following arrestin-dependent ATR uptake by OpsP in the rod disc membrane, subsequent uptake of the inverse agonist 11-*cis*-retinal (i.e., rhodopsin regeneration) is blocked in the receptor population that has already taken up agonist but not in the remaining receptor population with empty retinal binding pocket (Fig. 1d, e) (Sommer et al. 2012). This attribute allows each arrestin-bound receptor pair to simultaneously act as a sink for both ATR and 11-*cis*-retinal. Asymmetric ligand binding imposed by arrestin-1 would both protect the rod cell from free ATR and support continuous regeneration with 11-*cis*-retinal.

3.4 *Sequestered ATR is Still Accessible to Retinol Dehydrogenase*

Our proposed model of arrestin-mediated protection of the rod cell raises the question of how sequestered ATR eventually enters the retinoid cycle. Stimulation of retinol dehydrogenase (RDH) activity by the addition of its essential cofactor NADPH dissociates the ternary complex of arrestin, OpsP, and ATR (i.e., arrestin/Meta II-P) (Sommer et al. 2012). Several factors contribute to the efficiency of RDH-induced dissociation of the complex. First, with a reported K_M of ~1 μM (Palczewski et al. 1994), RDH binds ATR with higher affinity than arrestin-bound OpsP (K_D ~ 3–5 μM) (Sommer et al. 2012). Second, the ternary complex exists in equilibrium with its dissociated components. In practical terms, ATR is released every minute or two as Meta II decays.[2] Once released, ATR is available for enzymatic reduction by RDH. Since the reduction of ATR is essentially irreversible under cellular conditions, RDH effectively siphons away ATR from the arrestin/OpsP/ATR complex over time.

We hypothesize that arrestin-bound OpsP would serve a sink for ATR that cannot immediately be reduced by RDH (Fig. 1f). Thus, arrestin-dependent

[2] Based on the measured off-rate of ~0.01 s^{-1} for the arrestin-bound Meta II-P complex at physiological temperatures (Sommer et al. 2005).

sequestering would be most vital during those times when the bleaching rate outpaces ATR reduction. Indeed, Blakely et al. have reported that the rate of ATR reduction in isolated mouse rod cells is significantly slower than ATR release from isolated light-activated rhodopsin (Blakeley et al. 2011). In addition, Lee et al. observed in living mice that ATR levels increased faster than the level of its downstream products within the first 10 min after the onset of constant illumination (Lee et al. 2010). Furthermore, ATR reduction following significant bleaches can be impeded by limited NADPH concentration (Miyagishima et al. 2009).

While it is clear that sequestration of ATR cannot stop its entry into the retinoid cycle, the micromolar affinity of ATR for arrestin/OpsP should logically result in arrestin-dependent slowing of ATR reduction. Such an effect has been observed in vitro using isolated rod disc membranes (Hofmann et al. 1992; Palczewski et al. 1994). Interestingly, mouse rods lacking functional arrestin-1 showed no difference in the rate of all-*trans*-retinol formation (Blakeley et al. 2011), and arrestin-1 appeared to have little effect on the rate of 11-*cis*-retinal regeneration in live mice (Palczewski et al. 1999). However, these results do not necessarily contradict our proposed role for arrestin-1, because both studies employed a single bright illumination of short duration and then followed retinoid metabolism in the dark. Considering the majority of arrestin-1 is located in the inner segment in the dark-adapted rod (Strissel et al. 2006; Song et al. 2011), and arrestin-1 translocation to the outer segment takes many tens of minutes (Strissel et al. 2006), such a lighting protocol may minimize the effect of arrestin-1. Further experimentation, which tests the effect of constant illumination, is required to ascertain the influence of arrestin-1 on the enzymatic reduction of ATR in vivo.

3.5 Perspectives and Future Directions

While a lack of functional arrestin-1 in mice leads to significant light-dependent retinal damage (Chen et al. 1999; Hao et al. 2002; Burns et al. 2006), human patients suffering Oguchi disease due to a lack of functional arrestin-1 do not always suffer retinal damage (Paskowitz et al. 2006). It is likely that environmental factors, such as time spent in bright daylight, contribute to whether retinal degeneration accompanies Oguchi disease. In addition, other sinks for ATR may also exist and act in conjunction with arrestin-bound OpsP. These include the side product of Meta II decay, Meta III (Heck et al. 2003a), and secondary binding pockets within opsin (Heck et al. 2003b; Schädel et al. 2003). Even A2E formation has been proposed to protect the rod cell from free ATR (Wielgus et al. 2010), in opposition to the general belief that A2E is a toxic byproduct of vision (Sparrow et al. 2010).

Further work is required to examine how arrestin-1 influences the levels of toxic free-ATR in vivo. Of the many studies that have utilized arrestin-1 knockout mice over the years (Xu et al. 1997; Chen et al. 1999; Palczewski et al. 1999; Hao et al. 2002; Burns et al. 2006; Blakeley et al. 2011), none have examined how

arrestin-1 influences the levels of toxic free-ATR in the living animal exposed to realistic lighting conditions. The approach could be similar to that taken recently by Palczewski and colleagues in their studies of mice lacking RDH and the ABCA4 transporter (Maeda et al. 2009, 2011; Chen et al. 2012). Arrestin-1 knockout mice would be expected to show the same markers for oxidative stress and increased levels of retinal condensation products like A2E when exposed to bright continuous light. Furthermore, drugs containing primary amines would be expected to protect arrestin-1 knockout mice from bright light-induced retinal degeneration [see Maeda et al. (2011)].

4 Arrestin Translocation

In mice, arrestin-1 is expressed at 80 % the levels of rhodopsin (molar ratio) (Strissel et al. 2006; Hanson et al. 2007a). The majority of this arrestin-1 (estimated at 80–93 %) is sequestered in the rod inner segment in the dark-adapted rod cell (Strissel et al. 2006; Song et al. 2011). Due to its propensity to dimerize and tetramerize (Schubert et al. 1999; Hanson et al. 2007b), arrestin-1 might be confined to the inner segment because arrestin-1 oligomers cannot diffuse into the narrow spaces between the discs of the outer segment (Najafi et al. 2012). Inner segment confinement of arrestin-1 might be further enhanced by microtubule binding (Nair et al. 2004, 2005). The arrestin-1 present in the dark-adapted outer segment (about one arrestin-1 for every 10 rhodopsins) is monomeric (Hanson et al. 2007b; Najafi et al. 2012) and sufficient to quench signal transduction in dim light. Even lower levels of arrestin-1 (one arrestin-1 for every 200 rhodopsins) are sufficient to support normal photoresponse recovery following light flashes (Cleghorn et al. 2011). Exposure to bright continuous light triggers the translocation of the large pool of arrestin-1 from the rod inner segment to the outer segment over the course of many minutes (Elias et al. 2004; Strissel et al. 2006). Binding to Meta II-P is likely the driving force of translocation [see Slepak and Hurley (2008) and Gurevich et al. (2011)], although other gating and/or transport mechanisms may exist (Orisme et al. 2010). Following translocation, arrestin-1 interaction with phosphorylated receptor species, including Meta II-P and OpsP, maintains arrestin-1 in the outer segment. Importantly, the fact that the rod cell expresses such a large amount of arrestin-1, and this arrestin-1 is mobilized by light levels that by far exceed the operational range of the rod cell (Strissel et al. 2006), supports the idea of arrestin-1 as protector of the rod cell from the effects of brighter light. Evidence suggests that in moderate light, the protective effect of arrestin-1 is based on blocking transducin activation by Meta II and opsin (Hao et al. 2002), since persistent signaling by photoactivated and photo-decayed rhodopsin is deleterious to the rod cell and leads to apoptotis [reviewed in Fain (2006)]. In brighter light, arrestin-mediated protection is based not only on signal quenching (Hao et al. 2002) but probably also the limitation of toxic levels of ATR. In essence, arrestin-bound

OpsP buffers free ATR concentrations, which would be vitally important when the light suddenly intensifies and bleaches more rhodopsin.

Upon the return of the rod cell to darkness, arrestin-1 movement back to the inner segment is slow and follows the time-course of opsin dephosphorylation (Nair et al. 2005).

Moreover, receptor dephosphorylation follows the rate of rhodopsin regeneration (Lee et al. 2010), suggesting that full receptor deactivation by 11-*cis*-retinal uptake is required to dissociate arrestin-1 and return it to the inner segment. Hence the protective effects of arrestin-1 are maintained until the system has fully returned to its dark-adapted state.

5 Conclusions

Arrestin-1 exists at the intersection of three functional modules that compose the visual system (Fig. 1f). First to be discovered was the role of arrestin-1 as a terminator of rhodopsin signaling within the visual signal transduction module. Later, arrestin-1 was observed to make dramatic light-dependent migrations between rod cell segments, demonstrating how the protein translocation module facilitates the shift of the rod cell from photon-detection to survival mode. Finally, we describe a role for arrestin-1 in the retinoid cycling module, which helps the rod cell survive bright light by limiting levels of free ATR.

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Cone Arrestin: Deciphering the Structure and Functions of Arrestin 4 in Vision

Cheryl Mae Craft and Janise D. Deming

Contents

Abstract Cone arrestin (Arr4) was discovered 20 years ago as a human X-chromosomal gene that is highly expressed in pinealocytes and cone photoreceptors. Subsequently, specific antibodies were developed to identify Arr4 and to distinguish cone photoreceptor morphology in health and disease states. These reagents were used to demonstrate Arr4 translocation from cone inner segments in the dark to outer segments with light stimulation, similarly to Arrestin 1 (Arr1) translocation in rod photoreceptors. A decade later, the Arr4 crystal structure was solved, which provided more clues about Arr4's mechanisms of action. With the creation of genetically engineered visual arrestin knockout mice, one critical function of Arr4 was clarified. In single living cones, both visual arrestins bind to light-activated, G protein receptor kinase 1 (Grk1) phosphorylated cone opsins to desensitize them, and in their absence, mouse cone pigment shutoff is delayed. Still under investigation are additional functions; however, it is clear that Arr4 has non-opsin-binding partners and diverse synaptic roles, including cellular anchoring

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and trafficking. Recent studies reveal Arr4 is involved in high temporal resolution and contrast sensitivity, which opens up a new direction for research on this intriguing protein. Even more exciting is the potential for therapeutic use of the Arr4 promoter with an AAV-halorhodopsin that was shown to be effective in using the remaining cones in retinal degeneration mouse models to drive inner retinal circuitry for motion detection and light/dark discrimination.

Keywords Visual arrestins • Phototransduction • Gene regulation • Evolution • AAV-halorhodopsin

1 Discovery of Cone Arrestin

After "rod" Arrestin 1 (Arr1) and the two β-arrestins were identified (Attramadal et al. 1992; Lohse et al. 1990; Shinohara et al. 1987), the molecular search continued for other novel arrestins (Craft et al. 1990). The fourth arrestin was independently discovered using two distinct molecular cloning strategies. The first approach employed a technique that identified expressed retinal-specific genes on the X chromosome using a retinal cDNA library and northern blot screen analysis. Based on the sequence similarity to Arr1, this arrestin was named "X-arrestin" (Murakami et al. 1993). Simultaneously, Craft, Whitmore, and Wiechmann characterized the arrestin family using a pineal gland cDNA expression library by targeting an epitope domain-shared anchor of the three known arrestins in a novel polymerase chain reaction technology (PCR) approach (Craft et al. 1994). In addition to the known arrestins, they also identified a unique cDNA, which encoded an arrestin-like protein that was localized to human chromosome Xq13.1. Based on in situ hybridization studies, the transcript's cellular expression pattern demonstrated that it was highly enriched in pinealocytes and cone photoreceptors and was named "cone arrestin." Arrestin 4 (Arr4) is used to distinguish it from the other three arrestins (Craft et al. 1995).[1]

[1] Systematic names of arrestin proteins: Arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), Arrestin-2 (β-arrestin1 or ARRB1), Arrestin-3 (β-arrestin2, hTHY-ARRX or ARRB2), and Arrestin-4 (cone or X-arrestin; its human gene is designated "*Arrestin 3*" (ARR3) in the HUGO nomenclature database).

2 Cellular Localization and Expression

With the identification of the protein encoding Arr4, specific peptide antibodies were created that were helpful in resolving results from earlier immunohistochemical studies (Zhu et al. 2002a, b). Arr4 was shown to be specifically expressed in all cones (Zhang et al. 2001); however, it has reduced expression in S cones (Haverkamp et al. 2005). In contrast, a monoclonal antibody panel of S-antigen recognized Arr1expression in baboon rods and S-opsin cones, which was absent in LM-opsin cone photoreceptors (Nir and Ransom 1992). The same expression pattern is also observed in *Macaca*, chimpanzee, and human retinas (Zhang et al. 2001; Craft et al. 2013). In addition, these and other reagents are being used in numerous studies to identify cones in multiple species during development in normal retinas and retinoblastomas, cone survival in macula translocation, retinal degeneration models, and in cone rescue with gene therapy (Albini et al. 2004; Busskamp et al. 2010; Haire et al. 2006; Nikonov et al. 2006; Smith et al. 2000; Yu et al. 2011; Zhang et al. 2004).

By immunohistochemical localization using specific antibodies unique for mouse Arr4 (Luminaire junior—mouse cone arrestin [LUMIj-mCAR]), Arr4 is expressed in several cone photoreceptor cellular compartments before and after light exposure (Zhu et al. 2002a). Similar to Arr1 translocation studies in rod photoreceptors (Whelan and McGinnis 1988), Arr4 undergoes a light-dependent translocation from the cone pedicles, cell bodies, and inner segments to the cone outer segments. Similar light/dark Arr4 translocation is observed in bovine cone photoreceptors with the 7G6 monoclonal antibody, which also recognizes cone arrestin (Zhang et al. 2003a). However, the translocation of Arr4 is not as robust as that of Arr1; even after bright light exposure, a residual amount of Arr4 remains in the cone pedicle, while Arr1 nearly completely translocates to the outer segments (Zhu et al. 2002a). In $Grk1^{-/-}$ mice with or without simultaneous knockout of transducin α-subunit, Arr4 translocation to outer segments is light dependent, even without opsin phosphorylation (Zhang et al. 2003b). This implies that the classical "on" pathway through the opsins to alpha-transducin is not required for Arr4 translocation, and there is likely to be another light-dependent pathway driving the translocation of Arr4. It has also been shown that light-dependent Arr4 translocation does not take place in Guanylyl Cyclase 1 knockout ($GC1^{-/-}$) mice; however, Arr4 translocation can be restored when $GC1^{-/-}$ mice are treated with AAV-GC1, which rescues guanylyl cyclase 1 cone function (Coleman and Semple-Rowland 2005; Haire et al. 2006).

The concentration of the visual arrestins in dark-adapted cones was measured and compared to previous studies to reveal their combined total quantity is about 70 % of cone opsin (Nikonov et al. 2008). In this study in a single wild-type cone, Arr1estimated expression level was ~1.7×10^8 and Arr4 was ~3.3×10^6 molecules using whole cone volume of 950 ± 220 μm^3. Even more surprising, this quantitative analysis of immunofluorescence distribution of staining by Arr1-specific antibody D9F2 compared to Arr4-specific LUMIj-mCAR revealed differences in various cone compartments showing that Arr1 concentration is approximately 50-fold higher (Fig. 6.1).

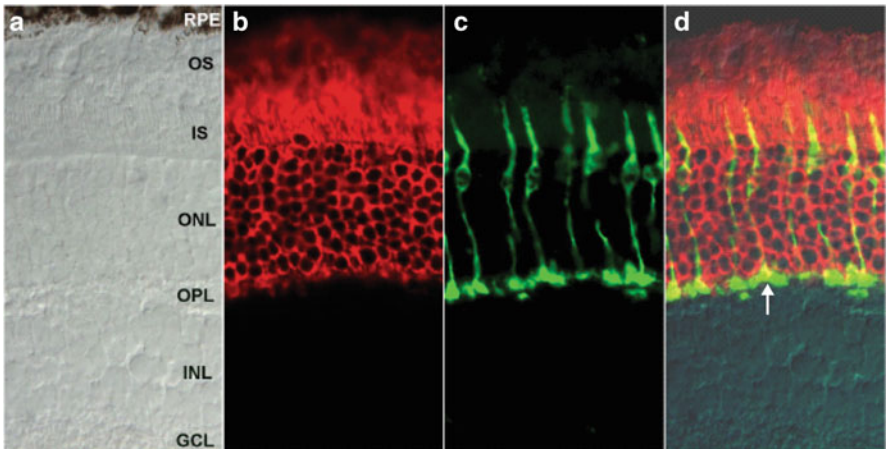

Fig. 6.1 Immunohistochemical staining of Arr1 and Arr4 in mouse cone photoreceptors. The four panels depict confocal images of a cryosection of a C57Bl/6J mouse retina with anti-mouse monoclonal D9F2-Arr1 (**b**) and anti-rabbit polyclonal LUMIj-mCAR (**c**) double labeled fluorescently with appropriate secondary antibodies (Zhu et al. 2002a). The overlay (**d**) reveals dual localization of Arr1 with Arr4 in the cone photoreceptor (*white arrow*). In panel (**a**), phase-contrast image is shown. Retinal pigment epithelia (RPE), outer segments (OS), inner segments (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), and ganglion cell layer (GCL)

3 Evolution of Cone Arrestin

The phylogeny of the vertebrate arrestins was summarized previously in an excellent review (Gurevich and Gurevich 2006). The visual and beta (β)-arrestins diverged from a family of ancient, alpha-arrestins and likely coevolved with the opsins (Alvarez 2008). Although Arr4 was so named because it was most recently discovered, it may not be the most recently evolved of the arrestins. With the understanding that early pineal photoreceptor cells were more similar to cones than to rods, it is likely that the cone arrestin emerged as the first member in the super family of the arrestins (Craft and Whitmore 1995). At least nine vertebrate species of Arr4 are in the NCBI database and range from *Danio rerio* to *Homo sapiens* (http://www.ncbi.nlm.nih.gov/homologene?cmd=Retrieve&dopt=MultipleAlignment&list_uids=3182). Phylogenetic analysis of visual arrestins suggests that Arr1 and Arr4 are likely to have diverged from *Ciona intestinalis* arrestin (Ci-Arr) around the same time (Nikonov et al. 2008). The Ci-Arr is expressed in ciliated, hyperpolarizing photoreceptors of the larval tunicate plus their axons and synaptic specializations (Horie et al. 2005) (Fig. 6.2). Based on the similarities, additional Arr4-binding partners in synaptic specializations of cones are a reasonable prediction.

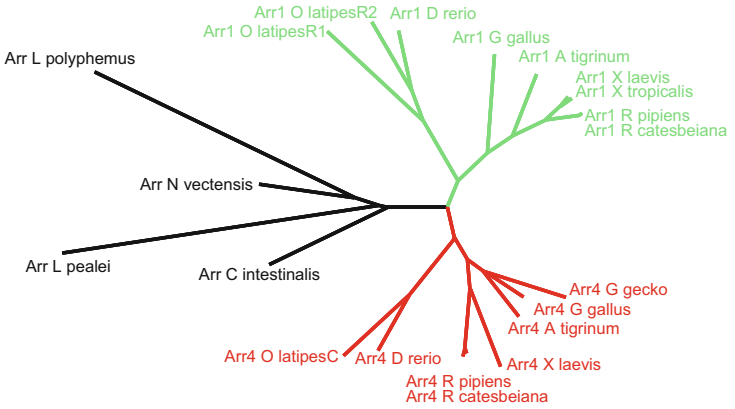

Fig. 6.2 Phylogenetic analysis of arrestin sequences from lower organisms. Primary sequences of arrestin proteins from the indicated species were aligned and an unrooted phylogenetic tree was generated using ClustalW (Thompson et al. 1994). Tree data were rendered with Dendroscope (Huson et al. 2007). Subtrees are color coded according to visual arrestin type (Arr1—*green*, *top*, Arr4—*red*, *lower*) where such designation has been reported; the branches in *black* (*middle*) correspond to species of identified visual arrestin sequences either not assigned or whose ancestors diverged from the line leading to vertebrates prior to the divergence of Arr1 and Arr4. R1, R2, and C designations for the *O. latipes* sequences are as described by the authors (Imanishi et al. 1999). Original from supplemental figure 9S (Nikonov et al. 2008) with permission for use granted by Elsevier: http://dx.doi.org/10.1016/j.neuron.2008.06.011 DOI:10.1016/j.neuron.2008.06.011#doilink

4 Crystal Structure and G Protein-Coupled Receptor Binding

Another critical piece of the puzzle in deciphering Arr4's functions was the generation of a crystal structure of the protein. In 2005, a crystal structure of the salamander cone arrestin was solved (Sutton et al. 2005). It was similar to the other arrestin structures that were previously identified, having the canonical arrestin fold consisting of two domains, each containing a β-strand "sandwich." The β-strand sandwich consists of two β-strand sheets joined by hydrophobic interactions. There was also a single α-helix in the amino (N)-domain. These investigators explored the binding selectivity of Arr4 compared to Arr1 and β-arr1. While Arr1 is highly selective for light-activated, phosphorylated-rhodopsin (P-Rh*), and β-arr1 is able to bind many G protein-coupled receptors (GPCRs), Arr4 has an intermediate binding selectivity. Its highest binding affinity was for human green cone opsin, but it was also able to bind to the M2 muscarinic cholinergic receptor. Thus, while the molecular structural details of Arr1 function have been well characterized, there is still much to discover regarding Arr4 and its function in cone photoreceptors and pinealocytes.

While the β-arrestins share a high sequence similarity (76 % identical), the visual arrestins are less similar to each other (58 % identical). Arr4 shares the same degree of similarity to β-arr2 as to Arr1 (58 %) (Craft and Whitmore 1995). Perhaps this similarity to the β-arrestins is what confers Arr4 with its binding capacity for GPCRs outside of the opsins, while Arr1 maintains a very high preference for P-Rh*.

5 Role in S- and M-Cone Opsin Shutoff

Given its amino acid sequence identity and similarity to Arr1 and enrichment in cone photoreceptors, researchers hypothesized that Arr4 acts in a similar physiological manner to Arr1: binding to light-activated, phosphorylated cone opsins and subsequently desensitizing them. This binding would prevent the phosphatase 2A from dephosphorylating the opsin complex and allowing it to be reactivated. However, until a decade after the initial discovery of Arr4, there was insufficient evidence to support this hypothesis. Because of the rod dominance of the mouse retina, it was difficult to isolate cone photoreceptors and determine whether or not the Arr4 was involved in cone pigment shutoff and how that involvement occurred. In 2001, using retinas isolated from the neural retina leucine zipper knockout mouse ($Nrl^{-/-}$), in which the rod progenitor cells develop into an enhanced S-cone phenotype, Swaroop and his collaborators observed high expression of cone-enriched proteins. Using immunoprecipitation, in vitro phosphorylation, and isoelectric focusing, Craft and her collaborators verified that Arr4 binding was specific to light-activated, G protein receptor kinase 1- (Grk1) phosphorylated S- and M-opsins in mice (Zhu et al. 2003; Mears et al. 2001). Backcrossing $Nrl^{-/-}$ with $Grk1^{-/-}$ mice to create double knockout mice, they revealed that when Grk1 is absent, the cone pigments are not phosphorylated and Arr4 is unable to bind them in a light-dependent manner. This was the first clear evidence that Arr4 acts in the way it had been hypothesized since its discovery. Additional in vitro studies suggested Arr4 participated in binding to light-activated phosphorylated cone opsins (Sutton et al. 2005; Zhu et al. 2002a). However, it still did not show that Arr4 is required for the cone pigment shutoff, but only that it binds to the cone opsins after they were light activated and subsequently phosphorylated by Grk1.

Craft and Pugh collaborated to clarify Arr4's contribution to cone pigment shutoff utilizing the Arr4 mouse knockout (Nikonov et al. 2008). To their surprise, their initial studies revealed no significant difference in the $Arr4^{-/-}$ cone pigment shutoff response compared to the control in native murine cones. An earlier study demonstrated that in a transgenic mouse model where cone arrestin expression was driven by the rhodopsin promoter to be highly expressed in rods that Arr4 could only partially rescue the light-induced rod degeneration and activated rhodopsin shutoff and recovery in $Arr1^{-/-}$ retinas (Chan et al. 2007).

Although Arr4 is expressed in cone photoreceptors and pinealocytes, Arr1 was discovered to be highly expressed in all mouse rods and co-expressed with Arr4 in cones (Nikonov et al. 2008; Zhu et al. 2005) (Fig. 6.1). They hypothesized that the

Arr1 may contribute to the cone pigment shutoff. Therefore, they employed single *Arr1*$^{-/-}$, *Arr4*$^{-/-}$, and double knockout *Arr1*$^{-/-}$*Arr4*$^{-/-}$ mice to determine if one or both visual arrestins were necessary and sufficient for normal cone pigment shutoff. Using electrophysiological recording from single cones of normal control mice, they showed that after a bright light stimulus, there is essentially no response difference in the cone recovery time between WT, *Arr1*$^{-/-}$, and *Arr4*$^{-/-}$. In contrast, *Arr1*$^{-/-}$*Arr4*$^{-/-}$ double knockout (*Arr*-DKO) response had a significantly longer recovery time compared to the single arrestin knockout genotypes. For the first time, this study established a function for Arr4 in living cones (Fig. 6.3, left panel).

Further experiments probed the time course of phototransduction activated by S- and M-cone opsins, respectively. Previously, it was shown that in a "dim-flash" response to 360 and 510 nm light, the response is a linear function of flash intensity and can independently be evaluated (Nikonov et al. 2006). Surprisingly, the *Arr*-DKO cones exhibited a similar waveform response to the other genotypes until they achieve 60 % of their recovery to baseline; then, the recovery response of the *Arr*-DKO cone "peeled off," exhibiting a much slower tail phase than the others, regardless of whether S- or M-opsin was activated by the flash (Fig. 6.3a–d, right panel). Therefore, the normal inactivation of each isomerized S- or M-opsin molecule requires at least one visual arrestin (Arr1 or Arr4) after a strong bright light stimulus. This avoidance of saturation in steady illumination implies that the phosphodiesterase activity generated by each photoisomerized cone opsin is prolonged. Thus, the current state of Arr4 research indicates that Arr4 binds to and desensitizes light-activated, phosphorylated cone pigments; however, Arr1 fulfills a similar functional role if Arr4 is absent.

6 Other Potential Binding Partners of Arr4 Are Identified

As with the other arrestins, Arr4 has other identifiable nonreceptor-binding partners, including *c-Jun* N-terminal kinase (Jnk3) and E3 ubiquitin ligase Mdm2. Arr4 works together with these proteins to regulate their subcellular localization and relocalize them from the nucleus to the cytoplasm (Song et al. 2007). Both of these proteins can also bind the other arrestins to serve as scaffolds to recruit modules (Lefkowitz and Shenoy 2005; Shenoy et al. 2001). Using a cell-based assay, Song and collaborators identified individual N- and C-domains of cone and rod arrestins that contain elements to bind JNK3 and to remove it from the nucleus. In contrast, unlike the interaction of the N-domain of Arr3, Mdm2 preferentially interacts with full-length Arr4 in the "frozen" basal configuration, which mimics the conformation of free Arr4. Their Arr4 studies exclude residues in the receptor-binding elements, plus set the stage to analyze the precise identification of Jnk3- and Mdm2-binding sites by site-directed mutagenesis.

In yeast two hybrid screens of retinal cDNA libraries, other potential interactions between Arr4 and novel candidates were identified, including Rnd2 (Zuniga 2010)

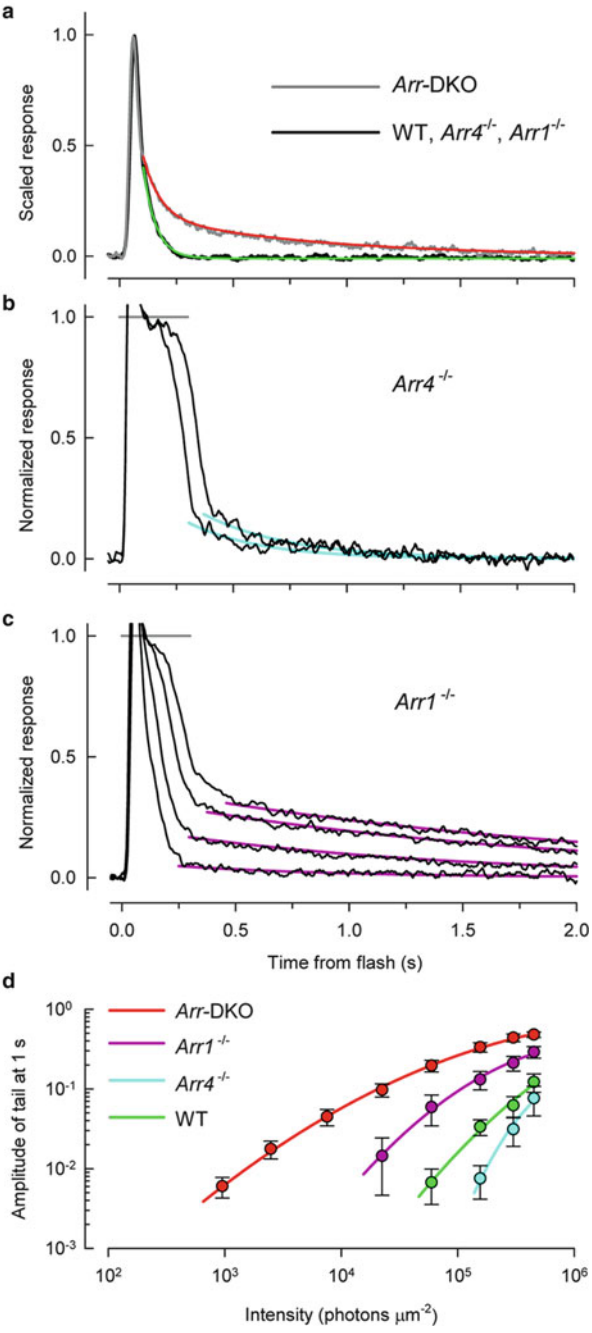

Fig. 6.3 (continued)

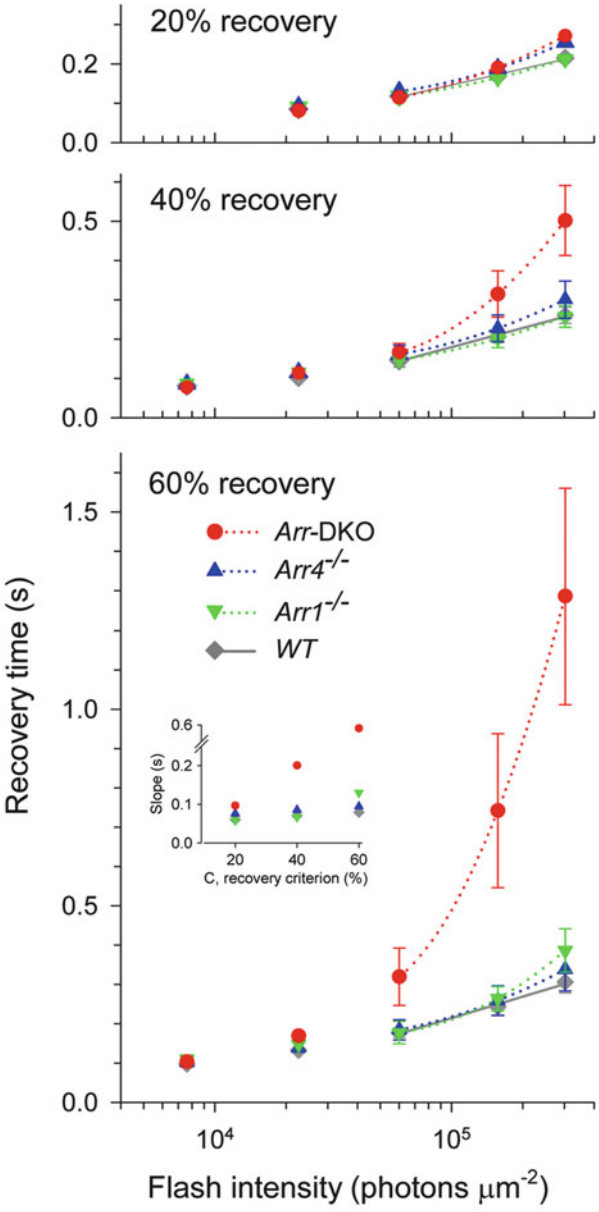

Fig. 6.3 Recovery times of S-dominant cones of WT, Arr4$^{-/-}$, Arr1$^{-/-}$, and Arr-*DKO* mice. The three panels are each Pepperberg plots, i.e., show as a function of the logarithm of the flash intensity the time T_C for cones of each genotype to recover criterion levels (C) of 20, 40, or 60 %, respectively, of their light-sensitive current after saturating flashes. The values at a set of discrete intensities were interpolated from individual cone's records and then averaged over genotype; the error bars are ±2 s.e.m. For *WT*, *Arr4*$^{-/-}$ and *Arr1*$^{-/-}$ cones the slopes of the "T_C vs log *I*" data are *roughly* constant across level C and genotype, in contrast, with the *Arr*-DKO data, for which the slope change strongly with C. These points are illustrated in the *inset* in the *lowermost panel* that plots the *Slopes* vs. C for each genotype. Original from figure 5 (Nikonov et al. 2008) and used

and a cilia protein, Als2Cr4/TMEM 237 (Zuniga and Craft 2010). Rnd2 belongs to a family of small GTP-binding proteins that alter many important cellular functions by affecting the actin cytoskeletal structure and stability (Tanaka et al. 2002). TMEM 237 is involved in the cilia transition zone and a gene defect contributes to Joubert syndrome (Huang et al. 2011) .

In addition, in an in vitro proteomic study in cultured HEK 293 cells, β-arrestins were shown to interact with both visual arrestins after stimulation with the beta-adrenergic agonist, isoproterenol (Xiao et al. 2007). So far, no evidence exists that the heteromerization of β-arrestins and visual arrestins has any functional significance, but they may work synergistically and in conjunction with one another, leading to an intriguing, unexplored area of inquiry (Deming et al. 2013).

Arr4 is highly expressed in cones and pinealocytes, and it is reasonable to hypothesize that it actively participates in other cellular interactions and other GPCR pathways besides cone opsin pigment shutoff, especially since pinealocytes do not express cone opsins. These interactions with other proteins could be responsible for the Arr4 remaining in the cone pedicle after light exposure.

7 Role in Visual Perception Phenotypes

Zebrafish studies have also provided evidence of the physiological role of Arr4 in vision. Zebrafish have two genes homologous to mouse Arr4, which are called Arr3a and Arr3b. Unlike mouse cones, which express both visual arrestins, zebrafish cone photoreceptors only express one visual arrestin per cone. M- and L-cones express Arr3b, while S-cones express exclusively Arr3a. Morpholino knockdown technology of Arr3b causes a delay in M- and L-cone photoreceptor recovery (Renninger et al. 2011). Because of technical limitations, S-cone photoreceptor recovery could not be measured, but the group predicted that Arr3a is required for S-cone recovery. In addition, Arr3b was shown to be necessary for high temporal resolution in the L- and M-cones (Renninger et al. 2011).

Fig. 6.3 (continued) with permission by Elsevier: http://dx.doi.org/10.1016/j.neuron.2008.06.011 DOI:10.1016/j.neuron.2008.06.011#doilink. (**a–d**) Response tail phases depend on visual arrestin genotype. (**a**) *Dim flash responses*. The *noisy black trace* presents the grand average dim-flash responses of cones that express only Arr4 ($Arr1^{-/-}$), only Arr1 ($Arr4^{-/-}$), or both arrestins (WT); the *noisy gray trace* is the averaged dim-flash response of *Arr*-DKO cones. Both averages combine S- and M-opsin driven responses, which had indistinguishable forms in each genotype. (**b–c**) *Responses to saturating flashes* of $Arr4^{-/-}$ cone and of $Arr1^{-/-}$ cone. The tail phases of the responses have been fitted with first-order exponential decays. (**d**) Summary analysis of the tail phase responses of all the cones investigated. The tail phase of each saturating response of every cone was fitted with exponential decays as in panel (**b**), (**c**), and the amplitude of the tail estimated from the fitted curve at $t = 1.0$ s after the flash; the values at a set of discrete intensities were interpolated and averaged over genotype. Original figure 8 (Nikonov et al. 2008) reproduced with permission granted by Elsevier: http://dx.doi.org/10.1016/j.neuron.2008.06.011 DOI:10.1016/j.neuron.2008.06.011#doilink

Mouse models utilizing the visual arrestin knockouts have a similar phenotype as the morpholino knockout of Arr3b. *Arr4*$^{-/-}$ mice have a significant decrease in contrast sensitivity compared to *Arr1*$^{-/-}$ or wildtype controls (Brown et al. 2012). Thus, although Arr1 can substitute for Arr4 in cone pigment shutoff, it may not be able to substitute all of the functional roles that Arr4 has in cones. Likewise, Arr4 expression will not substitute for Arr1 in restoring the light adaption ERG phenotype or synaptic modulation of *N*-ethylmaleimide sensitive factor in *Arr1*$^{-/-}$ (Brown et al. 2010; Huang et al. 2010). Other cone arrestin roles are under investigation, but the existence of the *Arr4*$^{-/-}$ mouse model will allow further discovery of the divergent cellular pathways in which the arrestins are involved.

8 Potential Therapeutic Use of Cone Arrestin Promoter

The mouse (mCAR) and human Arr4 (hCAR) gene spans over 13.5 kilobases (kb), which includes 17 exons and 16 introns (Sakuma et al. 1998; Zhu et al. 2002a). Similar to the other arrestins, mCAR also has alternative splicing with at least 5 transcripts. Both CAR promoters are well characterized and contain multiple *cis*-elements, including the cone rod homeobox (CRX), to regulate and target specific cone photoreceptor transcription. However, other specific promoter elements found in the mouse and human gene differ, including the AP4, c-Myb, and p53 elements in the former, and E-box, thyroid hormone/retinoic acid responsive, and derepression elements in the latter (Sakuma et al. 1998; Zhu et al. 2002a). These promoter elements were carefully studied in vitro in Y79 and WERI retinoblastoma cell lines and in vivo in *Xenopus laevis*. A region of less than 500 base pairs was shown to be necessary and sufficient to drive high levels of gene expression to a subpopulation of cultured retinoblastoma cells and cone photoreceptors and pinealocytes, respectively (Fujimaki et al. 2004; Li et al. 2002, 2003; Pickrell et al. 2004).

In 2010, the mCAR promoter was successfully used to target expression and to restore light-evoked activity in light-insensitive cone photoreceptors. Busscamp and colleagues genetically targeted a light-activated chloride pump, enhanced *Natronomonas pharaonis* halorhodopsin (eNpHR), to photoreceptors by means of adeno-associated viruses (AAVs) (Busskamp et al. 2010). Light-activated chloride pumps are rational candidates for reactivating vertebrate photoreceptors, as both eNpHR-expressing cells and healthy photoreceptors hyperpolarize in response to increases in light intensity. Two animal models of retinitis pigmentosa for gene therapy were tested. One of the targeted expression vectors of eNpHR was created with the use of the cell-specific promoter for mouse cone arrestin-4 (Zhu et al. 2002b). Virus was delivered after cones could not respond to light; however, the treated retinas could use their remaining inner retinal circuitry for motion detection and light/dark discrimination. Also, the NpHR did not elicit an immune response nor lead to toxicity after over 1 year.

The translation of gene therapy achieved in these mice to humans requires the use of promoters and AAV serotypes that drive photoreceptor-specific eNpHR

expression in human retinas. As part of the eNpHR studies, the AAVs were tested on human ex vivo retinal explants and they visualized eNpHR–EYFP protein expression in the cultured human retinas using the mCAR promoter-directed expression of eNpHR, which was specifically expressed in human photoreceptors (Busskamp et al. 2010). AAV vectors have proved to be stable and free of side effects when used to infect the human eye. The future hope is that AAV-halorhodopsin will be nontoxic and effective enough within the normal range of light intensities to prolong vision in humans with retinitis pigmentosa and perhaps other genetic diseases as well. The potential use of the AAV-halorhodopsin extends earlier work with gene therapy treatment with AAV-RPE65 of children with another form of genetic blindness, Leber's congenital amaurosis, which is currently approved (Testa et al. 2013). Alternatively, in the future a combination therapy of antioxidants, enzymes, and/or growth factors, and AAV-halorhodopsin might prolong cone survival and function (Cepko 2012). These exciting groundbreaking experiments that utilized the cone arrestin promoter are proof-of-principle examples toward realizing the therapeutic goal of restoring vision and demonstrate that expression and function of halorhodopsin in human cone photoreceptors are feasible.

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Enhanced Phosphorylation-Independent Arrestins and Gene Therapy

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Contents

Abstract A variety of heritable and acquired disorders is associated with excessive signaling by mutant or overstimulated GPCRs. Since any conceivable treatment of diseases caused by gain-of-function mutations requires gene transfer, one possible approach is functional compensation. Several structurally distinct forms of enhanced arrestins that bind phosphorylated and even non-phosphorylated active GPCRs with much higher affinity than parental wild-type proteins have the ability to dampen the signaling by hyperactive GPCR, pushing the balance closer to normal. In vivo this approach was so far tested only in rod photoreceptors deficient in rhodopsin phosphorylation, where enhanced arrestin improved the morphology and light sensitivity of rods, prolonged their survival, and accelerated photoresponse recovery. Considering that rods harbor the fastest, as well as the

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most demanding and sensitive GPCR-driven signaling cascade, even partial success of functional compensation of defect in rhodopsin phosphorylation by enhanced arrestin demonstrates the feasibility of this strategy and its therapeutic potential.

Keywords Congenital disorders • Gain-of-function mutants • GPCRs • Arrestin activation • Enhanced arrestins • Functional compensation • Gene therapy • Protein-based therapeutics

1 Disorders Associated with Defects in GPCR Phosphorylation and Excessive Receptor Activity

Congenital disorders fall into two broad categories. Many are associated with loss-of-function mutations in particular genes. As a rule, these disorders are recessive, because normal wild-type (WT) protein encoded by the second allele can do the job. Rare cases of haplo-insufficiency are the only exception, where we need both functional alleles to produce necessary amounts of the protein. Thus, in most cases the disease develops only when both alleles carry loss-of-function mutations (i.e., the patient is compound heterozygous). Conceptually gene therapy of these disorders is quite straightforward: a gene encoding fully functional protein needs to be delivered. Even though technically this is not easy, recent success of three independent clinical trials where gene encoding functional RPE65 was delivered to patients with Leber's congenital amaurosis carrying loss-of-function mutations in this protein (Cideciyan et al. 2008; Hauswirth et al. 2008; Maguire et al. 2008; Bainbridge et al. 2008) demonstrate the feasibility of this type of gene therapy.

Diseases caused by gain-of-function mutations present much greater challenge. They are dominant, as the effect of one allele encoding hyperfunctional protein cannot be alleviated by the second perfectly normal allele. Most importantly, there are no good strategies to address this type of disorders. One possible approach is to deliver a ribozyme specifically designed to destroy mutant mRNA without touching the normal one. It must be very effective against the mutant form, as even very low expression of overactive protein is harmful (Chen et al. 1995). However, in case of many missense or frame-shift mutations, mutant and normal mRNAs differ only by a single nucleotide. This makes designing a ribozyme, which is very effective against the mutant form yet highly selective, so that it does not destroy virtually identical normal mRNA, next to impossible. The only alternative approach proposed so far is compensational gene therapy. The strategy here is to design a mutant version of a protein interacting with the one affected by disease-causing mutation, with functional characteristics changed in such a way that it will compensate for the excessive activity of inherited mutant. For example, if mutant G protein-coupled receptor (GPCR) signals too much, redesigned arrestin that quenches this signaling

more effectively than WT form would normalize the signaling, shifting the balance in the cell closer to the norm.

Several human disorders are associated with excessive activity of GPCRs (Schöneberg et al. 2004). In some cases these are genetic, when one allele encodes a constitutively active receptor, or a form that cannot be shut off by the normal two-step mechanism employed by most GPCRs: phosphorylation of active receptor by G protein-coupled receptor kinases (GRKs), followed by arrestin binding to active phosphoreceptor. Several cases of *retinitis pigmentosa* (a form of retinal degeneration leading to complete blindness) are caused by mutations in rhodopsin that eliminate the sites phosphorylated in WT rhodopsin by GRK1 (rhodopsin kinase) (Apfelstedt-Sylla et al. 1993; Kim et al. 1993; Restagno et al. 1993). Upon activation by light these mutants effectively couple to visual G protein transducin, but their signaling cannot be quenched by GRK- and arrestin-mediated mechanism common in GPCR superfamily (Gurevich et al. 2011, 2012).

In other cases mutant receptors have GRK phosphorylation sites, but demonstrate higher than normal constitutive (ligand-independent) activity. Constitutively active mutant of PTH-PTHrP receptor causes Jansen-type metaphyseal chondrodysplasia (Schipani et al. 1995). Constitutively active mutants of TSH receptor cause toxic thyroid adenoma, multinodular toxic goiter, and autosomal dominant non-autoimmune hyperthyroidism (Paschke 1996; Khoo et al. 1999; Claus et al. 2005). Moreover, certain forms of cancer are caused by activating mutations in Gq-coupled GPCRs: ectopic expression of serotonin 1c receptor was shown to trigger malignant transformation (Julius et al. 1989), and Gq-coupled muscarinic receptors were found to act as agonist-dependent oncogenes (Gutkind et al. 1991). Interestingly, Gq-coupled angiotensin receptor was first cloned as mas oncogene before the true identity of this protein was discovered (Jackson et al. 1988).

The signaling by most GPCRs is turned off by a conserved two-step mechanism: first, active receptor is phosphorylated by specific GPCR kinases (GRKs) (Gurevich et al. 2012), whereupon arrestin specifically binds active phosphoreceptor (Gurevich and Gurevich 2004). Bound arrestin covers the cytoplasmic tip of the receptor, thereby blocking its coupling to G proteins by steric exclusion (Wilden 1995; Krupnick et al. 1997). The mutation in a GPCR can eliminate GRK phosphorylation sites (Apfelstedt-Sylla et al. 1993; Kim et al. 1993; Restagno et al. 1993; Chen et al. 1995), so that mutant receptor is normally activated by an appropriate stimulus, but cannot be turned off by GRKs and arrestins (Chen et al. 1995). In many other cases the receptor is perfectly normal, and its excessive activity is the result of genetic or acquired signaling defects upstream, e.g., abnormally high levels of its activating endogenous agonist.

Regardless whether the original error is genetic or acquired, in all these cases the net result is essentially the same: excessive receptor signaling that leads to imbalances that underlie the disease. Thus, an arrestin with enhanced ability to quench the signaling by overactive GPCR has a good chance to compensate and bring the signaling balance closer to normal.

2 The Mechanism of Arrestin Activation by Receptor-Attached Phosphates

Mammals express four arrestin subtypes (Hanson et al. 2006b). Two are specialized visual: arrestin-1[1] is expressed at very high levels in rod (Strissel et al. 2006; Hanson et al. 2007a; Song et al. 2011) and cone (Nikonov et al. 2008) photoreceptors, whereas arrestin-4 is cone specific (Craft et al. 1994; Nikonov et al. 2008). The two nonvisual subtypes, arrestin-2 and arrestin-3, are ubiquitously expressed and regulate signaling by hundreds of different GPCRs (Gurevich and Gurevich 2006b). Structurally, all four vertebrate arrestins are very similar (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011): they are elongated (long axis ~75A) two-domain molecules with relatively few contacts between domains, one of which is the interaction of the C-tail coming back from the C-domain and interacting with two elements in the N-domain, β-strand I and α-helix I (Fig. 1). Numerous studies showed that the residues that directly interact with receptors are localized on the concave sides of both domains (Gurevich and Benovic 1993; Ohguro et al. 1994; Gurevich et al. 1995; Pulvermuller et al. 2000a; Dinculescu et al. 2002; Vishnivetskiy et al. 2004; Hanson et al. 2006a; Hanson and Gurevich 2006; Vishnivetskiy et al. 2011; Gimenez et al. 2012c) (Fig. 2). As could be expected in a protein that preferentially binds phosphorylated GPCRs, arrestins have numerous positively charged phosphate-binding residues, all but one of which are conserved in the family: two lysines in β-strand I (Lys-14,15; Lys-10,11, Lys-11,12; and Lys-6,7 in arrestin-1, -2, -3, and -4, respectively), and two lysines and two arginines in β-strand X (Gurevich and Benovic 1995, 1997; Vishnivetskiy et al. 2000) (Fig. 2). Arg-18 in the loop between β-strands I and II (Fig. 2) is only present in the most phosphorylation-dependent family member, arrestin-1 (Sutton et al. 2005), whereas in others there are uncharged residues in equivalent position (Pro-14, Pro-15, and Ser-10 in arrestin-2, -3, and -4, respectively).

In all arrestins an unusual (for a soluble protein) arrangement of five virtually solvent-excluded charged residues is found in the inter-domain interface (Fig. 3), which was termed the polar core (Hirsch et al. 1999). It includes two positive charges (Arg-175 and -382; Arg-169 and -393; Arg-170 and -392; Arg-165 and -370 in arrestin-1, -2, -3, and -4, respectively) and three negative charges (Asp-30, -296, and -303; Asp-26, -290, and -297; Asp-27, -291, and -298; Asp-22, -287, and -294 in arrestin-1, -2, -3, and -4, respectively). One of the positive charges, Arg175/169/170 in arrestin-1/2/3, directly binds receptor-attached phosphates (Gurevich and Benovic 1995, 1997). The neutralization or reversal of this charge by mutagenesis yields arrestin mutants that bind active non-phosphorylated forms of their cognate receptors with high affinity (Gurevich and Benovic 1995, 1997;

[1] Different systems of arrestin names are used in the field and in this book. We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called "arrestin 3" in the HUGO database).

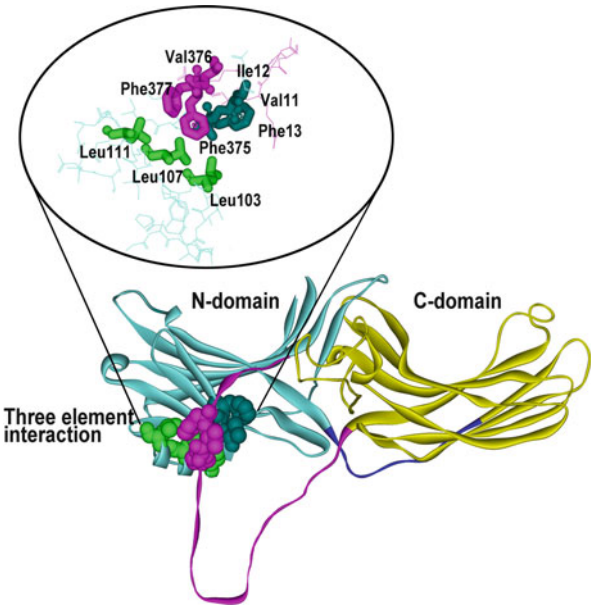

Fig. 1 Three-element interaction. Arrestins are elongated molecules consisting of the N-domain (*light blue*), C-domain (*yellow*), connected by a 12-resiude hinge (*dark blue*), and the C-tail (*magenta*). One of the interactions stabilizing the basal arrestin conformation involves bulky hydrophobic residues in b-strand XX in the C-tail (Phe375, Val376, Phe377, *magenta*), which comes back and interacts with b-strand I (Val11, Ile12, Phe13, *dark blue*) and a-helix I (Leu103, Leu107, Leu111, *green*) in the N-domain. The structure of arrestin-1 [1CF1 (Hirsch et al. 1999)] where this arrangement was first discovered is shown, but this structural feature is conserved in all arrestins (Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011). In all arrestins, destabilization of this interaction by triple alanine substitution of the hydrophobic residues in the C-tail (3A mutation) results in receptor binding-independent release of the C-tail and yields enhanced mutants that bind with higher affinity phosphorylated and non-phosphorylated active forms of their cognate receptors

Gray-Keller et al. 1997; Gurevich et al. 1997; Kovoor et al. 1999b; Vishnivetskiy et al. 1999; Celver et al. 2002a; Pan et al. 2003). The reversal of the negative charge of Asp296, which forms the salt bridge with Arg175, yields essentially the same enhancement of phosphorylation-independent binding to active receptors (Hirsch et al. 1999; Vishnivetskiy et al. 1999). Interestingly, simultaneous reversal of both charges, which restores the salt bridge in opposite configuration, fully restores strict dependence of arrestin binding on receptor phosphorylation (Vishnivetskiy et al. 1999). These results suggest that this salt bridge in the polar core is the main phosphate sensor in arrestins. Its disruption by negatively charged receptor-attached phosphates, which can occur regardless of the configuration of the bridge, turns arrestin "on," allowing its transition into high-affinity receptor-binding state (Gurevich and Gurevich 2004). Breakup of this salt bridge by mutations essentially "tricks" arrestin into perceiving any active form of the receptor as phosphorylated. Obviously, purely ionic mechanism of the phosphate sensor action only requires the presence of spatially concentrated negative charge on the receptor and therefore is

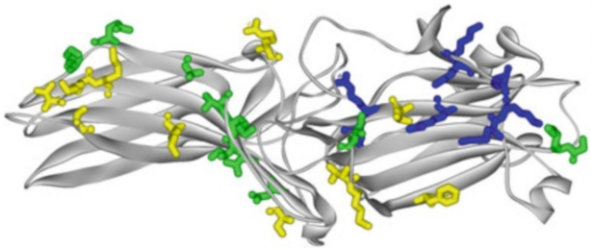

Fig. 2 Receptor-binding residues in arrestins. Crystal structure of arrestin-1 [1CF1 (Hirsch et al. 1999)] viewed from the concave side of both domains. Side chains of receptor-binding residues shown as *stick models*, color coded, as follows: *dark blue*, positive charges engaged by receptor-attached phosphates (Lys14, Ly15, Arg18, Lys166, Lys167, Arg171, Arg175, Lys176) (Gurevich and Benovic 1995, 1997; Vishnivetskiy et al. 2000; Sutton et al. 2005); *green*, residues that affect receptor selectivity of arrestins (Gly54, Lys55, Ile72, Val244, Asn246, Ile256, Lys257, Thr258, Ala261, Gln265, Lys267) (Vishnivetskiy et al. 2011; Gimenez et al. 2012c); *yellow*, other receptor-binding residues identified by site-directed mutagenesis (Gurevich and Benovic 1997; Hanson and Gurevich 2006) or site-directed spin labeling and EPR of arrestin-1 (Hanson et al. 2006b) or arrestin-2 (Vishnivetskiy et al. 2011) (Val74, Met75, Phe85, Leu173, Lys232, Thr233, Lys235, Lys236, Arg288, Lys330, Thr344). Homologous residues in other arrestin subtypes play the same roles

independent of the sequence context of phosphorylated serines and threonines. This simple mechanism of arrestin activation explained for the first time how two nonvisual arrestins in vertebrates (and only one in insects) can specifically bind active phosphorylated forms of hundreds of different GPCR subtypes (Gurevich and Gurevich 2006b).

It appears that by engaging the two lysines in the β-strand I receptor-attached phosphates also destabilize another key intramolecular "clasp" that holds arrestin in the basal conformation, three-element interaction of the C-tail, β-strand I, and α-helix I (Fig. 1) (Vishnivetskiy et al. 2000, 2010). Interestingly, these lysines are necessary for high-affinity binding of WT arrestin to active phosphoreceptor, but not for the binding of "pre-activated" mutants with either polar core or three-element interaction destabilized by mutations (Vishnivetskiy et al. 2000). Thus, it appears that highly exposed lysines on β-strand I "meet" receptor-attached phosphates first and then "guide" them to buried Arg-175 in the polar core, where they can destabilize this main phosphate sensor (Vishnivetskiy et al. 2000; Gurevich et al. 2011). Therefore, when the phosphate sensor is already turned "on" by mutagenesis, the job of the phosphates is done, making these "guiding" lysines in arrestin dispensable.

3 Construction of Enhanced Phosphorylation-Independent Arrestins

The main phosphate sensor, the polar core, is artificially turned "on" by mutations that disrupt the key salt bridge from either side, charge reversals of the Arg-175 and Asp-296 being virtually equipotent (Gurevich and Gurevich 2004). The fact that the

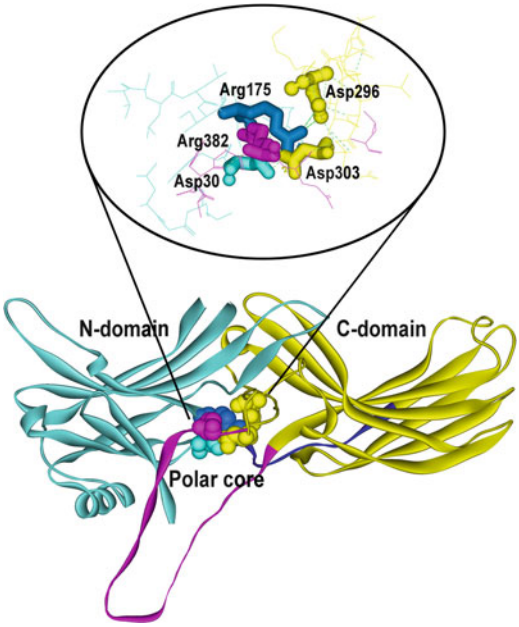

Fig. 3 The polar core—key phosphate sensor in arrestins. Arrestins are elongated molecules consisting of the N-domain (*light blue*), C-domain (*yellow*), connected by a 12-resiude hinge (*dark blue*), and the C-tail (*magenta*). Basal arrestin conformation is also stabilized by the network of ionic interactions on the inter-domain interface among five residues: three negatively charged Asp30 (from the N-domain, *light blue*), Asp 296, and Asp303 (from the C-domain, *yellow*) and two positively charged, Arg175 (from the N-domain, *dark blue*) and Arg382 (from the C-tail, *magenta*). The enraged image on *top* is rotated to better show all five residues involved. The structure of arrestin-1 [1CF1 (Hirsch et al. 1999)] where the polar core was first discovered and got its name is shown, but this structural feature is conserved in all arrestins (Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011). The polar core, particularly the salt bridge between Arg175 and Asp296, serves as the phosphate sensor: negatively charged receptor-attached phosphates break this salt bridge, which promotes arrestin transition into high-affinity receptor-binding state. The disruption of the polar core by mutations neutralizing or reversing the charge of Arg175 or Asp296 (or homologous residues in other arrestin subtypes) yields enhanced phosphorylation-independent mutants than bind with high-affinity active forms of their cognate receptors regardless of phosphorylation

disruption of the polar core, which supports the basal conformation in all arrestins (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011), pre-activates them, facilitating the binding even to non-phosphorylated receptors, is consistent with the idea that receptor binding is associated with a global conformational change in arrestin (Schleicher et al. 1989; Palczewski et al. 1991; Gurevich and Benovic 1993). It turns out that mutational disruption of the three-element interaction of the C-tail, β-strand I, and α-helix I, induces similar changes in the conformational equilibrium (Carter et al. 2005) and also greatly increases arrestin ability to bind active non-phosphorylated GPCRs (Gurevich 1998; Kovoor et al. 1999b; Celver et al. 2001, 2002a; Pan et al. 2003). One of the positive charges

in the polar core, Arg-382/393/392 in arrestin-1/2/3, is localized in the C-tail near the three-element interaction. However, forcible detachment of the C-tail by substitution of three bulky hydrophobics anchoring it to the body of the molecule (Fig. 1) with alanines (3A mutation) was found to be a more potent activating mutation that elimination of the positive charge of Arg-382 or its equivalent in nonvisual arrestins (Gurevich 1998; Kovoor et al. 1999b; Celver et al. 2002a). Interestingly, the deletion of the C-tail beyond its point of contact with the β-strand I and α-helix I yields essentially the same level of phosphorylation-independent binding as its detachment by alanine substitution in all arrestins (Gurevich et al. 1997; Gurevich 1998; Celver et al. 2002a; Vishnivetskiy et al. 2013a, b, c).

Thus, there are at least three types of mutations capable of significantly enhancing arrestin binding to active non-phosphorylated receptors (1) charge reversals of either Arg or Asp forming key salt bridge in the polar core; (2) detachment of the C-tail by substituting three bulky hydrophobic residues with alanines; and (3) deletion of the distal C-tail just beyond these bulky hydrophobics engaged in the three-element interaction. All yield enhanced arrestins with fairly high affinity for active receptors regardless of their phosphorylation. One limitation is that to be potentially useful for compensational gene therapy, mutant protein has to be stable, preferably as stable as parental WT one. One would expect any mutation that "loosens up" the basal conformation to reduce protein stability to some extent. This issue was systematically addressed in mouse arrestin-1 (Song et al. 2009; Vishnivetskiy et al. 2013a, b). Unexpectedly, it turned out that some of the activating mutations are a lot more detrimental for arrestin-1 stability than others (Song et al. 2009; Vishnivetskiy et al. 2013a, b). Charge reversals in the polar core greatly destabilize arrestin, whereas 3A mutation and C-tail deletion are fairly well tolerated (Song et al. 2009). In fact, even 3A variants of mouse arrestin-1 with additional mutations on the receptor-binding surface that increase their affinity for non-phosphorylated light-activated rhodopsin (Rh*) are fairly stable (Vishnivetskiy et al. 2013b) paving the way to the testing of these more potent pre-activated mutants in vivo.

Homologous mutations yield similarly enhanced phosphorylation-independent variants of nonvisual arrestins (Gurevich et al. 1997; Kovoor et al. 1999b; Celver et al. 2002a; Pan et al. 2003). These mutants effectively quench signaling by nonvisual GPCRs that are not phosphorylated either due to the loss of GRK sites or because GRKs are absent (Kovoor et al. 1999b; Celver et al. 2001, 2002a; Macey et al. 2006). Thus, enhanced nonvisual arrestins are perfectly ready for in vivo testing, but an additional serious issue needs to be addressed. Both nonvisual arrestins are promiscuous, comparably interacting with numerous GPCRs (Gurevich et al. 1995; Barak et al. 1997; Gimenez et al. 2012c). Virtually every cell in the body expresses 5–20 different GPCR subtypes. The expression of enhanced nonvisual arrestins will certainly suppress the signaling by hyperactive GPCR mutant. However, due to lack of receptor selectivity, enhanced versions of either arrestin-2 or -3 would dampen the signaling by all other perfectly normal GPCRs in the same cell, possibly doing more harm than good. Thus, practical use of enhanced nonvisual arrestins for gene therapy requires further work to significantly increase their receptor specificity (discussed in Chap. 8).

4 Compensational Approach to Gene Therapy

So far the ability of enhanced arrestin to compensate for defects in receptor phosphorylation was tested only in rod photoreceptors using a single model: rhodopsin kinase knockout (Song et al. 2009). Moreover, out of two stable enhanced mutants tested, mouse arrestin-1-3A and truncated arrestin-1-(1-377), only the former expressed at near-physiological levels in transgenic animals (Nair et al. 2005; Cleghorn et al. 2011; Song et al. 2011), and therefore only 3A mutant expressed at ~50 and ~220 % of WT arrestin-1, was actually tested for its compensational potential (Song et al. 2009). Considering that rods contain the fastest and the most sensitive GPCR-driven signaling cascade (Baylor et al. 1979; Gross and Burns 2010), which makes the visual system extremely demanding, this attempt was quite successful. Rod photoreceptors of rhodopsin kinase (GRK1) knockout mice (RKKO) rapidly lose their rhodopsin-containing signaling compartment, the outer segment, and then gradually degenerate (Chen et al. 1999; Song et al. 2009). The replacement of WT arrestin-1 in these rods with 3A mutant significantly improves their histological appearance and prolongs their survival (Song et al. 2009). Due to rod defect, RKKO animals demonstrate fairly low light sensitivity, effectively responding to brighter flashes that stimulate cones. In contrast, RKKO rods expressing 3A mutant at moderate level are functional and significantly more light sensitive (Song et al. 2009), again demonstrating certain level of compensation.

Since rhodopsin phosphorylation followed by arrestin-1 binding is critical for proper timing of the photoresponse (Xu et al. 1997; Chen et al. 1999; Mendez et al. 2000; Gross and Burns 2010), it was particularly important to test the effect of enhanced arrestin on the rate of photoresponse recovery. While WT rods rapidly restore sensitivity after moderately bright flashes, with time of half-recovery on the sub-second scale, RKKO rods recover extremely slow, with time of half-recovery ~18 s (Song et al. 2009). Good news is that the replacement of WT arrestin-1 with enhanced 3A mutant accelerated recovery about fivefold (Song et al. 2009), demonstrating that in principle compensational approach to gene therapy works even in the extremely demanding visual system. Bad news is that the recovery rate in "compensated" rods was still ~10-fold slower than in WT photoreceptors. Recent design of novel further enhanced mutants of arrestin-1 that bind to Rh* much better than the original 3A form and retain acceptable stability (Vishnivetskiy et al. 2013b) paves the way to testing the limits of this type of compensational gene therapy in rods.

It is entirely possible that the level of compensation achieved with 3A mutant would have been sufficient in any GPCR-driven signaling system that is less demanding than rod photoreceptors. In fact, in *Xenopus* oocytes mutants of arrestin-2 and -3 enhanced by homologous substitutions desensitize non-phosphorylated β2-adrenergic, μ- and δ-opioid receptors with virtually the same kinetics as WT arrestins in the presence of GRKs (Kovoor et al. 1999b;

Celver et al. 2001, 2002a). However, enhanced versions of nonvisual arrestins would only become therapeutically usable when their receptor specificity is narrowed to groups of receptors or even individual GPCRs (discussed in Chap. 8).

In some cases the mutation in a GPCR reduces arrestin binding to other functional forms than active phosphorylated receptor. Gly90Asp mutation in rhodopsin generates constitutively active form that causes night blindness in humans by desensitizing rods even in the dark (Sieving et al. 1995). This mutation impedes rhodopsin regeneration by retinal, because introduced aspartic acid forms a salt bridge with Lys 296 where retinal attaches (Singhal et al. 2013), so that a significant fraction of it exists as opsin that can activate transducin (Sieving et al. 1995). Interestingly, whereas WT phosphorylated opsin is the second highest affinity target of WT arrestin-1 (Sommer et al. 2012; Zhuang et al. 2013), phospho-opsin form of G90D mutant shows reduced arrestin-1 binding (Singhal et al. 2013). The same pre-activated arrestin-1 mutants that bind Rh* much better than WT, 3A, and truncated (Gurevich 1998; Song et al. 2009), demonstrate essentially normal binding to G90D phospho-opsin (Vishnivetskiy et al. 2013c), suggesting that reengineered arrestins have a potential to compensate for this type of defect, as well.

Importantly, even in case of arrestin-1, which until recently was believed to interact only with rhodopsin, other functional characteristics need to be taken into account. It was recently shown that even though arrestin-1 binds clathrin adaptor AP2 with affinity that is ~30 times lower than that of arrestin-2, excessive AP2 binding by rhodopsin-associated arrestin-1 can induce rod death (Moaven et al. 2013). AP2-binding site is localized in the arrestin C terminus (Kim and Benovic 2002), and the replacement of WT arrestin-1 in rods with its truncated form lacking AP2-binding site was shown to protect photoreceptors expressing constitutively active rhodopsin (Moaven et al. 2013). Also, arrestin-1-3A, in addition to apparently beneficial ability to bind Rh* (Song et al. 2009), is impaired in self-association (Song et al. 2013) (see Chap. 11). Resulting excessive concentration of monomer in mouse line expressing high level of this mutant appears to induce photoreceptor death via yet another mechanism (Song et al. 2013) (see Chap. 16). Collectively, these data suggest that to effectively compensate for defects of rhodopsin phosphorylation without unwanted side effects, enhanced form of arrestin-1 should not be able to bind AP2 and should either robustly self-associate or should be expressed at a relatively low safe level (Song et al. 2009, 2013).

5 Excessive Desensitization of Pre-activated Receptor Mutants and Normal Receptors Can Underlie the Pathology

Interestingly, in some cases constitutively active GPCRs are constitutively desensitized via hyper-phosphorylation by GRKs and virtually permanent association with cognate arrestin. For example, certain constitutively active rhodopsin mutants

were shown to be hyper-phosphorylated and associated with arrestin in vivo (Rim and Oprian 1995), suggesting that disease phenotype is just as likely to be caused by constitutive desensitization as by uncontrolled signaling. Another well-studied example is a naturally occurring R137H mutation in vasopressin receptor associated with familial nephrogenic diabetes insipidus. It was originally described as loss-of-function, but later mutant receptor was shown to be constitutively active, which leads to constitutive phosphorylation and arrestin binding in cells, resulting in receptor endocytosis and sequestration in the intracellular vesicles (Barak et al. 2001). Thus, this is another case where constitutive desensitization and internalization of overactive receptor gives an appearance of nonsignaling phenotype. It is entirely possible that some of the described loss-of-function GPCR mutations are in fact gain-of-function, but lead to constitutive desensitization in vivo.

Another well-established case where excessive desensitization plays critical role in pathology is congestive heart failure. Phenotypically, failing heart does not adequately respond to adrenergic stimulation, which is easily explained by decreased density of β-adrenergic receptors (Bristow et al. 1982). It has been shown that the expression of GRK2 is elevated in human failing heart and animal models of heart failure (Ungerer et al. 1993). High GRK2 activity results in excessive phosphorylation of β-adrenergic receptors and corresponding reduction in their responsiveness to sympathetic neurotransmitter norepinephrine and the adrenal hormone epinephrine. Importantly, the reduction of GRK2 activity toward β-adrenergic receptors by underexpression in hemizygous GRK2+/− mice or by transgenic expression of GRK2 C terminus that competes with endogenous GRK2 for G protein βγ subunits, thereby suppressing its recruitment to the plasma membrane where adrenergic receptors reside, restores receptor sensitivity to catecholamines, and improves heart function (Rockman et al. 1998a, b; Akhter et al. 1999).

6 Enhanced Arrestins Protect Receptor from Excessive Phosphorylation and Prevent its Downregulation

By virtue of binding to active non-phosphorylated receptors, enhanced phosphorylation-independent arrestin mutants compete with GRKs. Enhanced arrestin-2 mutant with disrupted polar core (R169E) was shown to suppress the phosphorylation of purified β2-adrenergic receptor by pure GRK2 in vitro, as well as in living cells (Pan et al. 2003). Interestingly, the expression of either arrestin-2-R169E or arrestin-2-3A (another enhanced mutant where the C-tail is detached) in cells was shown to prevent downregulation of β2-adrenergic receptor upon prolonged agonist stimulation (Pan et al. 2003). It was shown that in response to an agonist β2-adrenergic receptor is internalized equally rapidly in cells expressing WT arrestin-2 or enhanced mutant. The main difference was found to be in the rate of receptor recycling back to the plasma membrane, which was many times faster in mutant-expressing cells (Pan et al. 2003).

The mechanisms of GPCR cycling provide the simplest explanation of these findings. Under normal circumstances arrestin binding to the active phosphorylated receptor induces the release of the arrestin C-tail (Hanson et al. 2006b; Vishnivetskiy et al. 2010; Kim et al. 2012). This greatly increases the availability of clathrin and AP2-binding sites localized in this element of nonvisual arrestins (Goodman et al. 1996; Laporte et al. 1999; Kim and Benovic 2002), facilitating the recruitment of the arrestin–receptor complex to the coated pits. Internalized receptor is transported to endosomes, where its extracellular surface with bound agonist faces the lumen with relatively low pH. It is generally believed that this induces the release of bound agonist, which promotes receptor transition back to inactive state. Inactive phosphorylated receptors demonstrate 30–50 % lower arrestin binding than active phosphorylated forms (Gurevich et al. 1993, 1995; Kovoor et al. 1999a; Celver et al. 2002b), suggesting that receptor inactivation facilitates the release of arrestin. Since bound arrestin shields receptor-attached phosphates (Palczewski et al. 1989), arrestin dissociation is necessary to allow receptor dephosphorylation by cytoplasmic protein phosphatases, whereupon it becomes recycling competent (Morrison et al. 1996; Hsieh et al. 1999). The situation changes dramatically when the complex of enhanced arrestin with non-phosphorylated receptor is internalized. In this case receptor deactivation reduces arrestin binding manifold, rather than by a mere 30–50 % (Kovoor et al. 1999a; Celver et al. 2002b; Pan et al. 2003), suggesting that arrestin release would be much faster. Importantly, unphosphorylated and therefore fully recycling-competent receptor emerges immediately upon arrestin dissociation. These two factors acting together explain extremely rapid recycling of internalized β2-adrenergic receptor in cells expressing enhanced mutants (Pan et al. 2003). It appears that as it zooms through endosomes and back to the plasma membrane, receptor does not spend enough time in sorting endosomes to be diverted to lysosomes and degraded, which would explain how enhanced mutants protect the receptor from downregulation.

These data suggest that enhanced mutants of nonvisual arrestins can serve yet another purpose: protect the receptor from excessive phosphorylation and facilitate its recycling, which apparently prevents downregulation of the receptor. It appears that in situations associated with excessive phosphorylation and loss of the receptor, like congestive heart failure, this is likely to be beneficial. The ability of phosphorylation-independent arrestin mutants to protect heart function in conditions causing its failure needs to be tested experimentally.

7 Therapeutic Potential of Enhanced Visual and Nonvisual Arrestins

The success of the first proof-of-concept experiments in highly demanding visual system, where enhanced arrestin-1 partially compensated for the lack of rhodopsin phosphorylation, improving photoreceptor health, survival, and functional

performance (Song et al. 2009) demonstrates the feasibility of compensational gene therapy and its potential. However, neither morphology of "compensated" rods nor the rate of the recovery of their photoresponse was fully normalized: photoreceptors in WT animals outperformed compensated rods (Song et al. 2009). Thus, the challenge in this system is to construct more effective enhanced forms of arrestin-1, with much higher ability to bind non-phosphorylated light-activated rhodopsin and shut off its signaling. Several recent developments will facilitate progress in this direction. These include the analysis of rhodopsin binding-induced conformational changes in arrestin-1 using intramolecular distance measurements by pulse EPR (Kim et al. 2012), the identification of arrestin-1 elements engaged by different functional forms of rhodopsin using solution NMR (Zhuang et al. 2013), as well as the structures of truncated forms of arrestin-1 (Kim et al. 2013) and arrestin-2 (Shukla et al. 2013) that reveal the direction of the conformational changes in the process of arrestin activation by cognate receptors. Continuing improvements in engineered arrestin-1 mutants with phosphorylation-independent binding to active rhodopsin (Vishnivetskiy et al. 2013b) suggest that this goal is attainable. Next, the ability of new and improved enhanced mutant to compensate for impaired rhodopsin phosphorylation must be tested in different models of defective rhodopsin phosphorylation. In addition to GRK1 (rhodopsin kinase) knockout mice, where the previous mutant was tested (Song et al. 2009), compensation potential of new mutants should be tested in mice expressing rhodopsin without GRK1 phosphorylation sites, as well as those expressing rhodopsin with only one or two remaining sites (Mendez et al. 2000), which are insufficient for high-affinity arrestin-1 binding (Vishnivetskiy et al. 2007) in vitro and rapid rhodopsin shutoff in vivo (Mendez et al. 2000). Another unanswered question is whether these enhanced mutants should retain their ability to self-associate (see Chap. 11) or should be made constitutively monomeric (Hanson et al. 2008; Kim et al. 2011), since only arrestin-1 monomer can bind rhodopsin (Hanson et al. 2007b). Recent study showed that reduced self-association can be combined with enhanced phosphorylation-independent binding to active rhodopsin (Vishnivetskiy et al. 2013b). Optimal expression level of enhanced arrestin-1 is another issue that needs to be solved: previous experiments showed that the line expressing moderate (~50 % of WT) levels of enhanced arrestin-1 shows much better compensation that the line expressing it at more than twice WT level (Song et al. 2009). Moreover, progressive death of photoreceptors was documented in higher expressing line (Song et al. 2009). Since similarly high expression of WT arrestin-1 does not adversely affect photoreceptors (Song et al. 2011), this detrimental effect appears to be connected with mutation-induced changes in the molecule. Mutant-induced rod death was apparently associated with its impaired self-association that yields excessive concentration of arrestin-1 monomer (Song et al. 2013). Human homologue of the most promising form of enhanced mouse arrestin-1 that emerges from these experiments, expressed at optimal level, will be a good candidate to test for actual gene therapy in human patients. Thus, the challenge in the visual system, where it is clear that arrestin-1 subtype specifically

regulates rhodopsin signaling, is advanced engineering of a more powerful phosphorylation-independent mutant and precise determination of the range of expression levels that ensure safety and functional efficiency.

Other GPCR-driven signaling systems are not as sensitive as rod photoreceptors and demonstrate much slower shutoff kinetics (Carman and Benovic 1998; Violin et al. 2008). Phosphorylation-independent mutants of nonvisual arrestin-2 and -3 block G protein coupling of β2-adrenergic, μ-, and δ-opioid receptors yielding desensitization in the absence of receptor phosphorylation with essentially the same time course that WT arrestins yield in the presence of GRKs (Kovoor et al. 1999a; Celver et al. 2001, 2002b). Thus, it appears that there is no need to increase their efficiency, although homologues of some mutations on the receptor-binding surface of arrestin-1 with increased ability to bind non-phosphorylated receptors (Hanson and Gurevich 2006; Vishnivetskiy et al. 2013a, b) might further improve the performance of enhanced nonvisual arrestins. However, the main challenge with nonvisual arrestins is not efficiency, but receptor specificity. Both WT arrestin-2 and -3 are fairly promiscuous, binding comparably to numerous GPCRs (Gurevich et al. 1995; Barak et al. 1997), even though relative contribution of receptor-attached phosphates to arrestin binding varies widely in different cases (Gimenez et al. 2012a). Since most cells express a variety of receptors, only one of which would be mutant in each particular patient, the expression of enhanced versions of WT nonvisual arrestins would likely dampen the signaling by all GPCRs present in the same cell, instead of selectively suppressing faulty signaling by the mutant. Thus, to make enhanced nonvisual arrestins suitable for therapeutic purposes, it is imperative to increase their receptor specificity.

Receptor binding to any arrestin engages fairly large surface, covering most of the concave sides of both arrestin domains (Gurevich and Benovic 1993; Ohguro et al. 1994; Pulvermuller et al. 2000b; Hanson et al. 2006b; Hanson and Gurevich 2006; Vishnivetskiy et al. 2011). However, element swapping between arrestin-1 and -2 showed that only part of this extensive surface plays a role in receptor preference (Vishnivetskiy et al. 2004), and subsequent mutagenesis identified surprisingly few residues on it that define receptor specificity (Vishnivetskiy et al. 2011). These results, along with the fact that very few different amino acids occupied each of the key positions in arrestins from a variety of animal species from *Caenorhabditis elegans* to mammals (Gurevich and Gurevich 2006a), allowed the construction of a limited number of variants with point mutations in distinct receptor-discriminator sites (Gimenez et al. 2012b). Interestingly, out of the first 12 mutations tested on a set of 5 different GPCRs, 11 significantly affected receptor preference (Gimenez et al. 2012b). These results demonstrate that the construction of nonvisual arrestins with narrow receptor specificity is feasible (Chap. 8). Considering that the binding of phosphorylation-independent mutants to non-phosphorylated receptors tends to be more subtype specific (Kovoor et al. 1999a; Celver et al. 2002b), it is likely that enhanced versions of selective arrestins will retain narrow receptor specificity of parental mutants, although this still needs to be tested experimentally. If this turns out to be the case, enhanced

receptor-specific variants of arrestin-2 and -3 would be ready for in vivo testing of their ability to selectively suppress the signaling of only one of many GPCRs expressed in the same cell.

The ability of phosphorylation-independent arrestin-2 mutants to protect the receptor from excessive phosphorylation and downregulation was so far only demonstrated in cultured cells (Pan et al. 2003). If combining enhancing mutations with narrow receptor specificity proves feasible, these mutants should be tested for their ability to prevent phosphorylation and loss of an individual GPCR subtype among several in the same cell. The success of these experiments will provide reasons for testing the ability of receptor-specific versions of phosphorylation-independent nonvisual arrestins to protect β-adrenergic receptors in mouse models of heart failure and to improve heart function in these conditions. If these mutants work in living mice, as expected, this will pave the way for their therapeutic use for treating human patients with failing heart.

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Targeting Individual GPCRs with Redesigned Nonvisual Arrestins

Luis E. Gimenez, Sergey A. Vishnivetskiy, and Vsevolod V. Gurevich

Contents

Abstract Numerous human diseases are caused by excessive signaling of mutant G protein-coupled receptors (GPCRs) or receptors that are overstimulated due to upstream signaling imbalances. The feasibility of functional compensation by arrestins with enhanced ability to quench receptor signaling was recently tested in the visual system. The results showed that even in this extremely demanding situation of rods that have no ability to phosphorylate rhodopsin, enhanced arrestin improved rod morphology, light sensitivity, survival, and accelerated photoresponse recovery. Structurally distinct enhanced mutants of arrestins that bind phosphorylated and non-phosphorylated active GPCRs with much higher affinity than parental wild-type (WT) proteins have been constructed. These "super-arrestins" are likely to have the power to dampen the signaling by hyperactive GPCRs. However, most cells express 5–20 GPCR subtypes, only one of which would be overactive, while nonvisual arrestins are remarkably promiscuous,

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binding hundreds of different GPCRs. Thus, to be therapeutically useful, enhanced versions of nonvisual arrestins must be made fairly specific for particular receptors. Recent identification of very few arrestin residues as key receptor discriminators paves the way to the construction of receptor subtype-specific nonvisual arrestins.

Keywords Congenital disorders • Gain-of-function mutants • GPCRs • Receptor-specific arrestins • Functional compensation • Gene therapy • Protein-based therapeutics

1 The Case for Nonvisual Arrestins with High Receptor Specificity

The quenching of G protein-coupled receptor (GPCR) signaling was the first arrestin (arr) function described (Kühn et al. 1984; Lohse et al. 1990; Attramadal et al. 1992; Gurevich and Benovic 1995; Barak et al. 1997; Gurevich and Gurevich 2006b). After more than 30 years since rod arrestin (modern systematic name arrestin-1[1]) was first discovered (Kühn 1978; Kühn et al. 1984), receptor desensitization is still the best-characterized function of the members of this protein family. Vertebrate evolution created only one truly receptor-specific arrestin family member, visual arrestin-1, with high preference for rhodopsin (Gurevich et al. 1993, 1995; Vishnivetskiy et al. 2004, 2011), reasonable affinity for cone pigments (Sutton et al. 2005; Chan et al. 2007), and fairly low binding to nonvisual GPCRs (Gurevich et al. 2011). Even arr-4 expressed exclusively in cone photoreceptors (Craft et al. 1994; Nikonov et al. 2008) binds several GPCRs essentially as well as nonvisual arrestins (Sutton et al. 2005). Arr-1 is also the most selective: it binds to light-activated and phosphorylated rhodopsin (P-Rh*) with an affinity orders of magnitude higher than to non-phosphorylated light-activated (Rh*) or dark phosphorylated rhodopsin (P-Rh) (Gurevich and Benovic 1993; Zhuang et al. 2013). Arr-1 demonstrates high preference for P-Rh* over other GPCRs in vitro (Gurevich et al. 1993, 1995; Vishnivetskiy et al. 2004) and in live cells (Vishnivetskiy et al. 2011; Gimenez et al. 2012a). In contrast, nonvisual arrestins (arr-2 and arr-3 in vertebrates) are ubiquitously expressed and bind numerous GPCR subtypes (Gurevich et al. 1995; Barak et al. 1997; Gimenez et al. 2012b). Nearly 800 different genes encoding GPCRs have been identified in humans (Lagerstrom and Schioth

[1] Different systems of arrestin names are used in the field and in this book. We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called “arrestin 3” in the HUGO database).

2008; Almen et al. 2009; Nordstrom et al. 2011; Suwa et al. 2009) and the two nonvisual arrestins apparently bind most of these receptors (Gurevich and Gurevich 2006b). Although differences in the interactions between nonvisual arrestins and different receptors have led to a GPCR classification based on the stability of the complex (Oakley et al. 2000), the differences in arr-2 and arr-3 recruitment to various GPCRs do not create a significant selectivity that can be exploited experimentally or therapeutically (Vishnivetskiy et al. 2011; Gimenez et al. 2012a). Thus, if one intends to "tweak" the selectivity of nonvisual arrestins for different receptors, two questions must be answered. First, whether is it even possible to build into a nonvisual arrestin, high selectivity for a specific receptor? Second, in what context would arrestins with enhanced receptor selectivity be beneficial? These two questions define the scope of this chapter.

It is hard to overestimate the importance of GPCRs in general homeostasis. GPCRs are key receptors in most sensory systems, detecting light, odorants, and taste molecules. About 400 GPCRs in every mammal respond to hormones, neurotransmitters, and autacoids. Also known as seven-transmembrane domain receptors, or 7TMRs, GPCRs regulate a myriad of critical functions in unicellular and multicellular organisms (Dohlman et al. 1991). For example, yeast haploid cell types express Ste2 and Ste3, which respond to α and a-factor pheromones, promoting cell cycle arrest and fusion with cells of opposite mating type (Versele et al. 2001). Also in yeast, glucose triggers the shift towards the anaerobic conversion of the sugar into ethanol. This process is initiated by the activation of another GPCR, the glucose receptor Gpr1 (Kraakman et al. 1999).

In multicellular organisms, GPCR signaling is required to maintain homeostasis and to ensure coordinated cellular function. Novel functions of GPCRs are constantly being identified. For example, in *Drosophila melanogaster*, the product of *mth* encodes a secretin receptor-like GPCR called Methuselah (Mth). Mth regulates life span in flies (Lin et al. 1998) by modulating the oxidative stress resistance response (Araujo et al. 2013; Gimenez et al. 2013) through mechanisms that involve controlling secretion of insulin-like peptides from a restricted population of insulin-producing cells (IPCs) in the brain (Gimenez et al. 2013). Unexpectedly, both expression of dominant negative mutants of Mth and overexpression of this protein in the IPCs result in a prolonged fly life span (Gimenez et al. 2013). Thus, normal longevity is only observed when fly IPCs receive strictly calibrated signaling from Mth.

In vertebrates, GPCRs mediate constant hormonal control of organ function, as well as tissue growth and cell proliferation, during normal and pathological adaptation. In most cases, prolonged uncontrolled stimulation of any GPCR leads to pathology. In the heart, neuroendocrine stimulation initiated by cardiac adrenergic receptors induces hypertrophic changes of the myocardium (Dorn and Force 2005). Under persistent stimulation, excessive cardiac remodeling can lead to heart failure, as has been shown in a murine model of persistent muscarinic receptor stimulation by antibodies with agonist-like action (Gimenez et al. 2005). Agonist-like autoantibodies mediating prolonged receptor stimulation were found in patients with Chagas' disease and other dilated cardiomyopathies (Ribeiro et al. 2007;

Hernandez et al. 2008). Their deleterious effects highlight the importance of balanced GPCR signaling.

Several human disorders are caused by activating mutations in various GPCRs (Schipani et al. 1995; Paschke 1996; Khoo et al. 1999; Claus et al. 2005; reviewed in Schöneberg et al. 2004; Vassart and Costagliola 2011) or genetic errors eliminating GRK phosphorylation sites (Apfelstedt-Sylla et al. 1993; Kim et al. 1993; Restagno et al. 1993) necessary for timely signal shutoff (Chen et al. 1995). These gain-of-function mutations are dominant, i.e., the other allele encoding a normal protein cannot reduce the signaling by an overactive mutant. An even greater variety of disorders are associated with excessive GPCR signaling caused by pharmacological therapeutic interventions (Ahmed et al. 2010). It stands to reason that arrestins with greater than normal ability to quench GPCR signaling, which can be constructed in several ways (see Chap. 7), can functionally compensate (Song et al. 2009). It is very likely that when excessive GPCR signaling underlies the pathology, bringing the balance back to normal will cure the disease.

However, virtually every cell in the body expresses between 5 and 20 different GPCRs, only one of which is a mutant or signals too much for some other reason. Both nonvisual arrestins bind many GPCRs with similar affinity (Gurevich et al. 1995; Barak et al. 1997; Gimenez et al. 2012b), and activating mutations make them even less discriminating (Gurevich et al. 1997; Kovoor et al. 1999; Celver et al. 2002). Thus, an enhanced mutant constructed on the basis of promiscuous nonvisual arrestins will reduce the signaling by the overactive GPCR, while simultaneously dampening the signaling by all other receptors expressed in the same cell. This is likely to cause side effects that could be even worse than the disease itself. Thus, therapeutic use of enhanced nonvisual arrestins requires the construction of mutants with narrow receptor selectivity, better yet with a strict specificity for an individual GPCR subtype that needs to be targeted.

2 Identification of an Extensive Receptor-Binding Arrestin Surface

Before the discovery of the arrestin–clathrin interaction (Goodman et al. 1996), GPCRs were the only known class of arrestin-binding proteins. Considerable effort by many groups was invested into the identification of arrestin residues directly engaged by receptors and mapping of the receptor "footprint" on arrestin. In fact, many arrestin elements involved in receptor binding were identified before the first crystal structure became available (Gurevich and Benovic 1993, 1995, 1997; Gurevich et al. 1993, 1995; Ohguro et al. 1994; Gray-Keller et al. 1997). The residues identified in these studies were later mapped onto the structure of the basal conformation of bovine arr-1 (Granzin et al. 1998; Hirsch et al. 1999) and found to be localized on the concave sides of both arrestin domains.

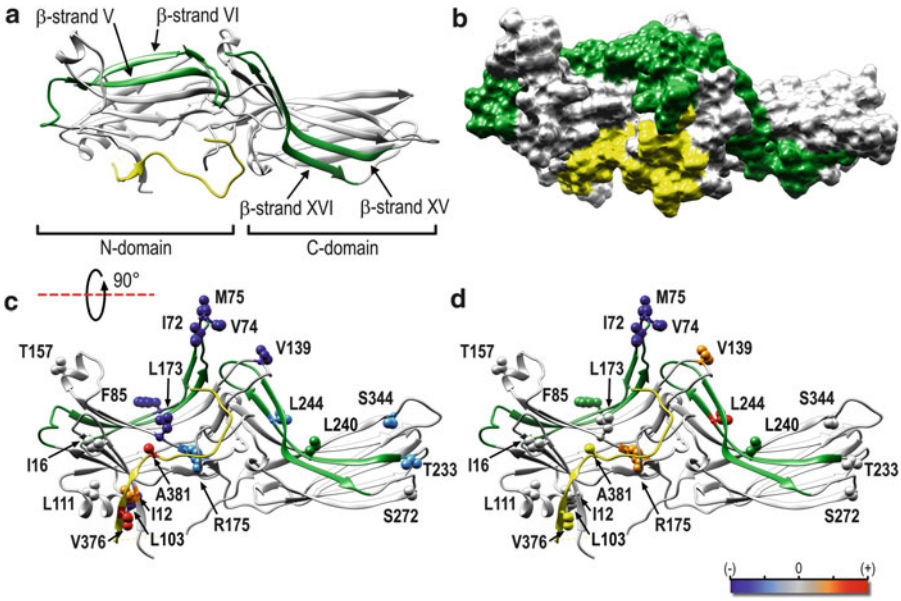

Fig. 1 The receptor-binding interface has been mapped to the concave side of both domains in all arrestin subtypes. (**a**) Ribbon representation of bovine arr-1 based on PDB: 1CF1 (Hirsch et al. 1999) as viewed from the receptor "viewpoint." Arrestins consist of two domains linked by a flexible hinge and the C-tail that comes back from the C-domain and makes a strong contact with the β-strand I and α-helix I in the N-domain (see Chap. 7, Fig. 1). The β-strands V-VI and XV-XVI with adjacent loops, identified as key elements that determine receptor specificity (Vishnivetskiy et al. 2004), are shown in *green*; the C-tail (including the parts not resolved in crystal structures) is shown in *yellow*. (**b**) Space-filling model of arr-1, oriented and color coded as in panel (**a**). (**c**, **d**) Side view of arr-1 [90° rotation from the perspective shown in panel (**a**)] with spin-labeled residues (Hanson et al. 2006) shown as *ball-and-stick models*. The magnitude of the detected changes in spin-label mobility upon receptor binding is color coded as follows: *gray* (or *green/yellow*), no change; *pink/red*, small and large increases in mobility, respectively; *light blue/dark blue*, small and large decreases in mobility, respectively. (**c**) Changes upon binding to dark (inactive) P-Rh. (**d**) Additional changes induced by light activation of P-Rh to P-Rh*. Upon binding to dark P-Rh (**c**), finger loop residues (I72, V74, M75) become less mobile, while the mobility of the C-tail residues increases. Light activation further decreases the mobility of the finger loop residues (**d**), while mobility of V139 increases [this loop was later shown to move out of the way of incoming receptors (Kim et al. 2012; Vishnivetskiy et al. 2013)]. Ribbon and surface cartoons rendered with UCSF Chimera 1.8

Interestingly, every arrestin element identified by subsequent studies using peptide competition (Pulvermuller et al. 2000), epitope insertion (Dinculescu et al. 2002), element swapping (Vishnivetskiy et al. 2004), site-directed mutagenesis (Vishnivetskiy et al. 1999, 2000, 2010, 2011; Hanson and Gurevich 2006), site-directed spin labeling/EPR (Hanson et al. 2006; Vishnivetskiy et al. 2010, 2011; Kim et al. 2012), and NMR (Zhuang et al. 2013) was also found to localize to the same concave sides of the two arrestin domains (Fig. 1). Thus, we can be fairly confident that regardless of the arrestin–receptor combination, the entire receptor

"footprint" is localized within these concave surfaces, and it likely covers a considerable fraction of them.

Existing data indicate that the receptor-binding arrestin elements likely include noncontiguous residues distributed through this surface of the protein. Each individual interaction between arrestins and receptors is relatively low-affinity, but simultaneous engagement of several elements yields a high-affinity complex (Gurevich and Benovic 1993; Krupnick et al. 1994). As a result, not all potential interaction sites on both partners need to be engaged to allow arrestin to perform its functions. The complexes held together by fewer elementary interactions would have reduced affinity and stability. This is the probable mechanistic basis of the functional differences between class B GPCRs that hold arrestins tightly and travel with them all the way to late endosomes (Oakley et al. 2000) and class A receptors that readily release bound arrestins upon internalization.

3 Few Arrestin Elements Determine Receptor Preference

Discrete interactions of individual arrestin residues distributed over an extensive receptor-binding surface were shown to account for receptor selectivity that determines arr-1 preference for rhodopsin, as well as preferential binding of nonvisual arrestins to other GPCRs. This was elegantly demonstrated in a study where multiple elements were swapped between arr-1 and arr-2 in an attempt to identify those that determine this specificity (Vishnivetskiy et al. 2004). In this study, the parts of arr-1 that increased arr-2 binding to P-Rh* and the parts of arr-2 that improved arr-1 binding to M2 muscarinic acetylcholine receptor were identified. It turned out that two elements encompassing residues 49–90 (β-strands V and VI with adjacent loops) in the N-domain and residues 237–268 (β-strands XV and XVI) in the C-domain of visual arr-1 and homologous elements in arr-2 are the key players in receptor preference (Vishnivetskiy et al. 2004). The exchange of these two elements between arr-1 and arr-2 completely reversed receptor specificity of these two subtypes (Vishnivetskiy et al. 2004).

Individual residues that determine receptor preference of arrestins were identified in a subsequent study (Vishnivetskiy et al. 2011). Due to high homology between arr-1 and -2, as few as 35 residues in the two elements that engage receptors are different, and only 22 of these differences represent nonconservative substitutions (Vishnivetskiy et al. 2004). An attempt to construct arr-2 with arr-1-like preference for P-Rh* demonstrated that only five arr-2 residues (Leu-68, Ser-86, Asp-240, Asp-259, and Thr-261) are the key in determining its receptor specificity, whereas nine additional residues (Leu-48, Glu-50, Arg-51, Tyr-238, Cys-242, Lys-250, Cys-251, Pro-252, and Met-255) play a supporting role (Vishnivetskiy et al. 2011). Moreover, alanine substitution of ten of these residues (four in the N-domain and six in the C-domain) completely blocked the binding of arr-1, arr-2, and arr-3 to all GPCRs tested, including P-Rh* (Vishnivetskiy et al. 2011; Gimenez et al. 2012a).

An interesting feature that distinguishes nonvisual arrestins from arr-1 is revealed by the comparison of the crystal structures of arr-2 and arr-1 (Hirsch et al. 1999; Han et al. 2001). Each arrestin domain is a β-strand "sandwich," in which the two β-sheets are "glued" together via hydrophobic interactions between the side chains pointing inside the sandwich (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011). In visual arr-1, Val90 is one of these residues, participating in multiple interactions with hydrophobic side chains of Val45, Val57, Val59, and Phe118 (Hirsch et al. 1999). In nonvisual arrestins, this valine is absent, being replaced with serine (arr-2) or alanine (arr-3) (Han et al. 2001; Zhan et al. 2011). Even though all its potential partners are conserved in arr-2 (Val41, Val53, Val55, and Phe115), the absence of this valine apparently makes the N-domain more flexible. In contrast to arr-2, arr-1 demonstrates relatively low binding to active phosphorylated M2 muscarinic receptors (Han et al. 2001; Vishnivetskiy et al. 2004). The Val90Ser mutation in arr-1, which apparently "loosens up" the N-domain, dramatically reduces its preference for P-Rh*, enhancing the binding to M2 receptors (Han et al. 2001). The magnitude of the effect of the mutation of this one residue (the side chain of which is not even exposed) strongly suggests that a relatively rigid N-domain stabilized by the interactions of Val90 with its partners is an important contributor to the high specificity of arr-1 for P-Rh* (Han et al. 2001; Vishnivetskiy et al. 2004). In fact, the Val90Ser substitution increases arr-1 binding to active phosphorylated M2 muscarinic receptors more than any other point mutation reported (Han et al. 2001; Vishnivetskiy et al. 2011).

This proof-of-concept protein engineering highlights the importance of the insight provided by the availability of high-resolution structural data. It also suggested that any mutants of nonvisual arrestins designed for increased receptor specificity must have Val (found in the two visual subtypes, arr-1 and arr-4) (Hirsch et al. 1999; Sutton et al. 2005) in the equivalent position. It seemed reasonable to expect that on this rigid background predisposed to be receptor selective, substitutions of relatively few residues that determine receptor preference (Vishnivetskiy et al. 2011) would yield nonvisual arrestins with enhanced receptor specificity.

4 Construction of Nonvisual Arrestins with Increased Receptor Specificity

This approach was used to create a set of mutants on arr-3-Ala87Val background specifically intended for the generation of variants with high receptor specificity (Gimenez et al. 2012b). Arr-3 was used in this study because it was reported to be even more promiscuous than arr-2, capable of binding numerous GPCRs (Barak et al. 1997; Kohout et al. 2001; Zhan et al. 2011).

The Val87Ala mutation per se had negligible impact on arr-3 binding to M2 muscarinic and D1 and D2 dopamine receptors and slightly increased the binding to

β2 adrenergic receptor (β2AR) (Gimenez et al. 2012b). The next study focused on ten exposed residues, four in the N-domain and six in the C-domain, that were previously identified as critical for the receptor–arrestin interaction (Vishnivetskiy et al. 2011; Gimenez et al. 2012a). However, if one considers all possible permutations, where each position can be occupied by 20 different amino acids, the number of possible combinations is 20^{10} (i.e., more than 10 trillion), which is too large for experimental testing. However, the analysis of known arrestin sequences (Gurevich and Gurevich 2006a) shows that only two to three different residues were found in equivalent positions in arrestins from *Caenorhabditis elegans* to mammals. The logical assumption that amino acids that are never found in a particular position should not be there narrows the number of possible combinations down to manageable. Evolutionary sequence analysis (Gurevich and Gurevich 2006a) shows that the residues affecting receptor preference (Vishnivetskiy et al. 2011; Gimenez et al. 2012a) are actually islands of variability within highly conserved elements. Replacement of arr-3 residues only with those that naturally occur in equivalent positions in arrestins from other species virtually eliminates the possibility of misfolding.

The substitutions following this logic were introduced into eight out of these ten positions (Gimenez et al. 2012b). The recruitment of the generated arr-3 mutants to agonist-activated M2, D1, D2, and β2AR (Gimenez et al. 2012b) was measured using bioluminescence resonance energy transfer (BRET) between GPCRs tagged with *Renilla* luciferase on the C terminus and arrestins N-terminally tagged with Venus, a version of GFP (Namkung et al. 2009b; Walther et al. 2010). Interestingly, none of the mutations appreciably increased arr-3 binding to any of the receptors tested. However, seven out of ten significantly reduced the interaction with some of the receptors, but not with others, changing the selectivity up to fourfold (Gimenez et al. 2012b). This unexpectedly high ~70% success rate clearly shows that the key players in receptor specificity were correctly identified (Vishnivetskiy et al. 2011). This notion was further supported by the finding, with the use of direct in vitro binding assay with P-Rh* (Gurevich and Benovic 1992, 1993), that most substitutions significantly affected the ability of arr-3 mutants to interact with this model receptor (Gimenez et al. 2012b). Importantly, the combination of two mutations that significantly reduced β2AR binding without affecting the interactions with M2 and D2 receptors (Asp260Lys + Gln262Pro) yielded an arrestin with ~50-fold preference for these receptors over the β2AR (Gimenez et al. 2012b). Similarly, the combination of two substitutions that reduced the binding to D2, but not D1 receptors (Tyr239Thr + Gln256Tyr), generated an arrestin with more than fivefold preference for the D1 over D2 receptor (Gimenez et al. 2012b). Thus, the effects of individual mutations appear to be additive, which demonstrates the feasibility of the construction of nonvisual arrestins with high specificity for particular GPCRs (Fig. 2).

In-cell analysis of the binding of these arr-3 mutants to different GPCRs yielded yet another interesting finding. The arrestin–receptor interactions were found to have two distinct components: a basal, agonist-independent and an agonist-induced, each accounting for about half of the maximum observed binding (Gimenez

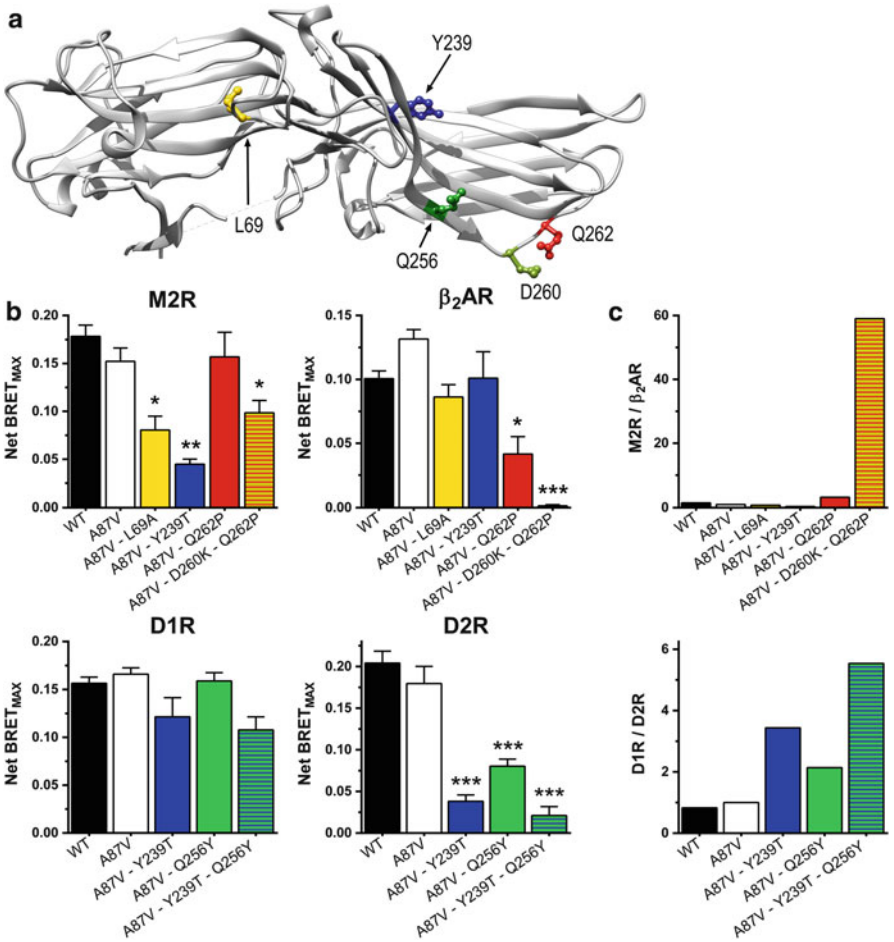

Fig. 2 Mutations of few residues increase the selectivity of arr-3 for certain GPCRs. (**a**) The residues on the receptor-binding surface of bovine arr-3 that affected receptor selectivity the most (Gimenez et al. 2012b) are shown as *ball-and-stick models*. (**b**) The effect of these mutations and their combinations (on the Ala87Val background) on agonist-induced arr-3 recruitment (Net $BRET_{max}$) to M2 muscarinic (M2R), D1 (D1R) and D2 (D2R) dopamine, and β2-adrenergic (β_2AR) receptors. (**c**) Ratios of net $BRET_{MAX}$ [shown in panel (**b**)] for the indicated mutants and receptor pairs are shown. For normalization, the binding ratio of the Ala87Val base mutant was set at 1. Asp260Lys + Gln256Tyr increased arr-3 preference for M2R over β_2AR to >50-fold, whereas Tyr239Tre + Gln256Tyr increased arr-3 preference for D1R over D2R to approximately fivefold

et al. 2012a, b). Interestingly, the manipulation of the receptor-binding surface changed these two components in the same direction to a similar extent, which was reflected in a very good correlation between mutation-induced changes in both basal banding and its agonist-induced increase (Gimenez et al. 2012b). Thus, a limited set of exposed residues mediates both the basal and agonist-induced arrestin

binding to GPCRs, and targeted mutagenesis of these elements is a feasible approach for the generation of inherently selective nonvisual arrestins specifically targeting individual receptor subtypes.

Arrestin mutants that combine narrow receptor specificity with increased ability to desensitize GPCRs that cannot be phosphorylated or have excessive activity for other reasons are likely to be effective tools for normalizing GPCR signaling in conditions where excessive signaling underlies the pathology. This promising research direction is still in its infancy, and a lot of additional work needs to be done to generate receptor-specific arrestins with high therapeutic potential.

5 Differential Role of Receptor-Attached Phosphates in the Binding of Different Arrestins

As a rule, arrestins preferentially bind active phosphorylated forms of their cognate receptors. The main phosphorylation sensor in all arrestins is structurally similar: the polar core, localized between the two arrestin domains, includes two positively charged arginines and three negatively charged aspartates (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011) (see Chap. 7, Fig. 3). Usually, in soluble proteins, charged residues are exposed on the surface, whereas the polar core in arrestins is buried. An arginine in β-strand X (Arg175, Arg169, or Arg170 in arr-1, arr-2, or arr-3, respectively) directly binds the phosphates attached to the intracellular loops and/or C terminus of GPCRs by GRKs (Gurevich and Benovic 1993, 1995, 1997; Granzin et al. 1998; Vishnivetskiy et al. 1999; Celver et al. 2002; Gurevich and Gurevich 2006b; Hanson and Gurevich 2006). Neutralization or reversal of the charge of this arginine by appropriate mutations artificially turns the phosphate sensor "on," greatly increasing arrestin binding to unphosphorylated active forms of their cognate receptors: Rh* in case of arr-1 (Gurevich and Benovic 1995, 1997; Gray-Keller et al. 1997; Gurevich 1998; Vishnivetskiy et al. 1999) or various nonvisual receptors in case of arr-2 and arr-3 (Gurevich and Benovic 1993; Gurevich et al. 1997; Kovoor et al. 1999; Celver et al. 2001, 2002; Pan et al. 2003; Schattauer et al. 2012). Each arrestin has numerous lysines and arginines that bind receptor-attached phosphates: several in β-strand X and preceding loop (Gurevich and Benovic 1995) and two lysines in β-strand I (Vishnivetskiy et al. 2000; Shukla et al. 2013) (see Chap. 7, Fig. 2). Arr-1 has an additional phosphate-binding residues, Arg19 in the loop between β-strands I and II (Sutton et al. 2005), which explains why arr-1 is more dependent on receptor-attached phosphates than nonvisual subtypes (Vishnivetskiy et al. 2000; Gimenez et al. 2012a; Kim et al. 2012; Zhuang et al. 2013). Interestingly, this remains true even in case of arr-1 binding to non-cognate receptors (Gimenez et al. 2012a, b). As far as nonvisual GPCRs are concerned, the role of receptor-attached phosphates varies widely, depending on a particular arrestin–GPCR combination (Mukherjee et al. 2002; Kim et al. 2004; Namkung et al. 2009a; Gimenez et al. 2012a) [see

also Chap. 2 and Gurevich and Gurevich (2006b) for review]. Using BRET between receptor-RLuc and Venus-arrestin it was recently shown that in case of the β2AR, phosphates play an important, although not as decisive role as in arr-1 binding (Gimenez et al. 2012a). As for the M2 muscarinic and D2 dopamine receptors, the role of phosphorylation in arrestin recruitment (Gimenez et al. 2012a) and signaling regulation (Namkung et al. 2009b) appears to be minimal, even though the phosphorylation of a particular cluster of serines and threonines in the third intracellular loop of M2 was shown to enable arrestin binding (Pals-Rylaarsdam et al. 1997; Lee et al. 2000). In all arrestin subtypes mutations that destabilize the polar core or delete or forcibly detach the C-tail displaced by receptor binding yielded "pre-activated" enhanced nonvisual arrestins that readily interact with cognate GPCRs in a phosphorylation-independent manner (Gurevich et al. 1997; Kovoor et al. 1999; Celver et al. 2001, 2002; Pan et al. 2003).

An enhanced phosphorylation-independent mutant of arr-1 was shown to compensate for the lack of rhodopsin phosphorylation in vivo, prolonging the survival and improving functional performance of rod photoreceptors (Song et al. 2009) (see Chap. 7). Enhanced nonvisual arrestins were shown to effectively shut off the signaling by several unphosphorylated GPCRs in cells (Kovoor et al. 1999; Celver et al. 2001, 2002) and in vivo (Bruchas et al. 2006). However, nonvisual arrestins are inherently promiscuous (Gurevich et al. 1995; Barak et al. 1997; Kohout et al. 2001; Gimenez et al. 2012b), and activating mutations make them even more flexible (Carter et al. 2005), so that the expression of phosphorylation-independent nonvisual arrestins in any cell, in addition to the desired suppression of the signaling by overactive receptors, would likely also dampen the signaling by other GPCRs present in the same cell, causing serious side effects. Thus, therapeutic use of enhanced nonvisual arrestins will be feasible when activating mutations are combined with those that narrow down their receptor specificity, preferably to small groups of receptors or individual GPCRs.

6 Usefulness of Arrestins with Greater Specificity for Individual Receptors

Overactive GPCRs cause signaling imbalances leading to disease via different mechanisms: excessive stimulation of a normal receptor by a ligand (Hernandez et al. 2003, 2008; Ribeiro et al. 2007; Stavrakis et al. 2009, 2011; Ahmed et al. 2010), activating mutations (Schipani et al. 1995; Paschke 1996; Schöneberg et al. 2004; Vassart and Costagliola 2011), or aberrant desensitization (Apfelstedt-Sylla et al. 1993; Kim et al. 1993; Restagno et al. 1993; Chen et al. 1995; Rim and Oprian 1995; Barak et al. 2001; Moaven et al. 2013). The development of enhanced nonvisual arrestins targeting a specific malfunctioning receptor holds promise of compensation with a potential of bringing the signaling closer to normal. Recent advances in the development of gene delivery methods suitable for therapy

(Ishikawa et al. 2011; Bartel et al. 2012; Nguyen and Szoka 2012; Dalkara et al. 2013) make the introduction of protein-based tools feasible (see chapter "Therapeutic potential of small molecules and engineered proteins").

Controlling runaway GPCRs is not the only potential therapeutic use of reengineered arrestins with narrow receptor specificity. In addition to shutting of G protein-mediated signaling (Carman and Benovic 1998), arrestins recruit GPCRs to coated pits for internalization via direct binding to clathrin (Goodman et al. 1996) and AP2 (Laporte et al. 1999) and initiate the second round of signaling by recruiting various non-receptor partners (Gurevich and Gurevich 2006a; DeWire et al. 2007). New generations of GPCR agonists biased towards G proteins or arrestins are becoming increasingly available (see chapter "Arrestin-biased GPCR agonists") with some currently tested in clinical trials for the treatment of pain and control of elevated blood pressure and even food intake (Reiter et al. 2012; Kenakin and Christopoulos 2013). Signaling-biased arrestin mutants with disabled individual functions, such as the ability to bind clathrin/AP2 (Kim and Benovic 2002) or MEK1 (Meng et al. 2009) and activate ERK1/2 (Coffa et al. 2011) or JNK3 (Seo et al. 2011; Breitman et al. 2012), are also becoming available. These designer arrestins equipped with additional mutations that make them specific for particular GPCRs can be used for selective channeling of arrestin-mediated signaling to desired pathways, while excluding unwanted ones. In combination with conventional or biased agonists, these arrestins can also be used to enhance traditional pharmacological therapy and make it more targeted. Phosphorylation-independent arrestin mutants were shown to support rapid internalization and recycling of GPCRs, preventing receptor downregulation (Pan et al. 2003). In several pathological conditions, such as congestive heart failure, excessive desensitization and downregulation of β-adrenergic receptors is at the root of the disease (Rockman et al. 1998). Arrestin mutants that can selectively prevent downregulation of β-adrenergic receptors have a potential to improve the performance of the failing heart.

Arrestins modulate an amazing variety of physiological processes, from GPCR trafficking (chapters "Arrestin interactions with G protein-coupled receptors" and "Arrestin binding to clathrin, AP2, and role in GPCR trafficking"), MAP activity (chapters "Arrestin-dependent activation of ERK and Src Family kinases", "Arrestin-dependent activation of JNK family kinases", and "Arrestin-mediated activation of p38 MAPK: molecular mechanisms and behavioral consequences"), cell motility (chapter "Molecular Mechanisms underlying beta-arrestin-dependent chemotaxis and actin-cytoskeletal reorganization") and heart function (Rockman et al. 1998) to aging (Gimenez et al. 2013). In most cases, arrestin interactions with particular GPCRs are responsible for these effects, both normal and pathological. Thus, nonvisual arrestins combining strict receptor specificity with different types of signaling bias have many potential therapeutic uses.

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Part II
Arrestin Interactions with Non-receptor Partners and Roles in Cell Signaling

β-Arrestins and G Protein-Coupled Receptor Trafficking

Xufan Tian, Dong Soo Kang, and Jeffrey L. Benovic

Contents

Abstract Nonvisual arrestins (β-arrestin-1 and β-arrestin-2) are adaptor proteins that function to regulate G protein-coupled receptor (GPCR) signaling and trafficking. β-arrestins are ubiquitously expressed and function to inhibit GPCR/G protein coupling, a process called desensitization, and promote GPCR trafficking and arrestin-mediated signaling. β-arrestin-mediated endocytosis of GPCRs requires the coordinated interaction of β-arrestins with clathrin, adaptor protein 2 (AP2), and phosphoinositides. These interactions are facilitated by a conformational change in β-arrestin that is thought to occur upon binding to a phosphorylated activated GPCR. In this review, we provide an overview of the key interactions involved in β-arrestin-mediated trafficking of GPCRs.

Keywords Arrestin • Receptor • Phosphorylation • Endocytosis • Clathrin • Adaptin • Phosphoinositides

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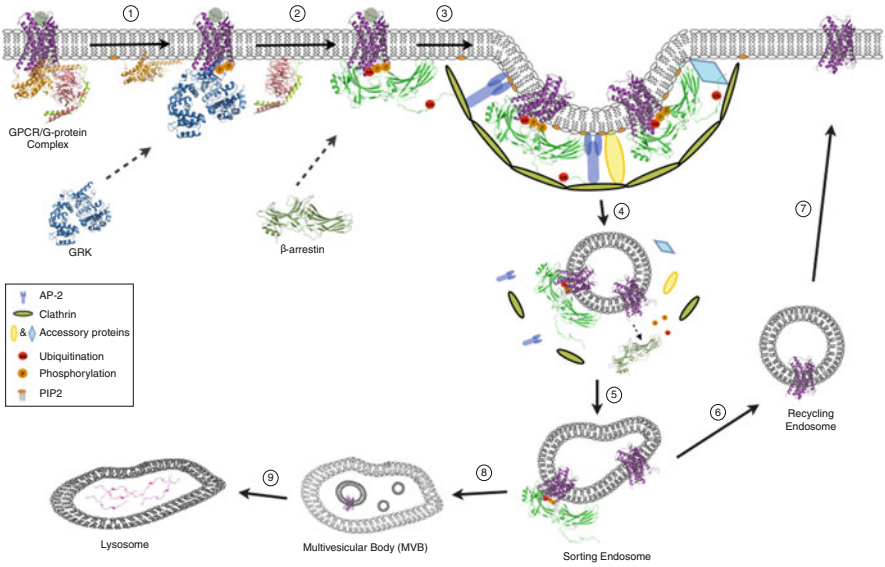

Fig. 1 Role of β-arrestins in GPCR trafficking. (1) Agonist binding to a GPCR results in heterotrimeric G protein activation leading to dissociation of Gα from Gβγ subunits. Receptor activation also promotes GRK association with the GPCR, which mediates receptor phosphorylation and (2) promotes β-arrestin recruitment to the receptor. (3) β-arrestin association with the phosphorylated GPCR mediates conformational changes in arrestin that promote association of the GPCR-β-arrestin complex with the endocytic machinery and subsequent endocytosis (4). GPCRs then traffic to sorting endosomes (5) and ultimately either are recycled back to the plasma membrane through recycling endosomes (6 and 7) or are sorted to lysosomes where they are degraded (8 and 9)

1 β-Arrestins and GPCR Trafficking

Many transmembrane signaling systems consist of specific G protein-coupled receptors (GPCRs) that transduce the binding of extracellular stimuli into intracellular signaling. GPCRs modulate the activity of numerous intracellular effectors and ultimately regulate a myriad of biological processes. To ensure that extracellular stimuli are translated into intracellular signals of appropriate magnitude and duration, most signaling cascades are tightly regulated. GPCRs are subject to three principal modes of regulation: (1) desensitization, where a receptor becomes refractory to continued stimuli; (2) internalization, where receptors are physically removed from the cell surface by endocytosis; and (3) downregulation, where total cellular receptor levels are decreased (Fig. 1). GPCR desensitization is primarily mediated by second messenger-dependent kinases and by GPCR kinases (GRKs). GRKs specifically phosphorylate activated GPCRs and initiate the recruitment of arrestins, which mediate receptor desensitization, endocytosis, and signaling (Krupnick and Benovic 1998).

A role for β-arrestins in agonist-promoted internalization of GPCRs was first discovered in 1996 (Ferguson et al. 1996; Goodman et al. 1996). These initial studies focused on the β_2-adrenergic receptor (β_2AR), while more recent studies have demonstrated that β-arrestins promote the trafficking of many GPCRs, as well as additional classes of receptors (Moore et al. 2007; Shenoy and Lefkowitz 2011). Mechanistic insight into this process has revealed an essential role for the coordinated interaction of β-arrestins with the GPCR (Vishnivetskiy et al. 1999, 2011), clathrin (Krupnick et al. 1997; Kang et al. 2009), adaptor protein 2 (AP2) (Laporte et al. 1999, 2000; Kim and Benovic 2002; Schmid et al. 2006; Burtey et al. 2007), and phosphoinositides (Gaidarov et al. 1999; Milano et al. 2006). Moreover, β-arrestin binding to the GPCR appears to induce a conformational change that promotes interaction with the endocytic machinery, thereby linking the binding and trafficking events (Kim and Benovic 2002; Xiao et al. 2004; Nobles et al. 2007).

2 General Structure of β-Arrestins

The four mammalian arrestins fall into two classes, visual and nonvisual, and X-ray structures for all four family members have been solved (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005; Zhan et al. 2011) (see Chap. 7, Figs. 1 and 3). Arrestins can be divided into two major domains, the N-domain and C-domain, with each domain primarily consisting of antiparallel β-sheets connected by short flexible loops (Fig. 2). The N- and C-domains are connected by a short "hinge region," while the C-tail is connected by a flexible linker to the C-domain and contains a short β-strand that interacts with a lateral β-strand of the N-domain. The overall structure is stabilized by a polar core of buried salt bridges and by a three-element interaction involving the first β-strand, an α-helix in the N-domain, and the C-terminal tail (Han et al. 2001; Milano et al. 2002, 2006; Kang et al. 2009; Zhan et al. 2011). The polar core is comprised of charged residues from the amino terminus (Asp-29 in β-arrestin-1), N-domain (Arg-169), C-domain (Asp-290 and Asp-297), and C-terminal tail (Arg-393), thus bringing different parts of the molecule together to maintain a basal conformation. The residues involved in formation of the polar core are highly conserved, suggesting that this structural element is critical for the function of all arrestins. Because the buried side chains of the polar core achieve neutrality by an elaborate network of electrostatic interactions, it has been suggested that disturbance of the polar core by introduction of a phosphate group from the receptor promotes structural changes that result in an active conformation of arrestin (Hirsch et al. 1999). Indeed, two of the five polar core residues, namely, Arg169 and Asp290 in β-arrestin-1 (Han et al. 2001) and Arg170 and Asp291 in β-arrestin-2 (Zhan et al. 2011), are particularly important for arrestin selectivity for binding to activated phosphorylated receptors (Vishnivetskiy et al. 1999).

It is believed that arrestins make an initial contact with phosphorylated receptors via adjacent lysines in the amino terminus (Vishnivetskiy et al. 2000). Biochemical data suggests that this interaction perturbs the three-element interaction, guides phosphorylated receptors to the polar core, allows the negatively charged phosphate

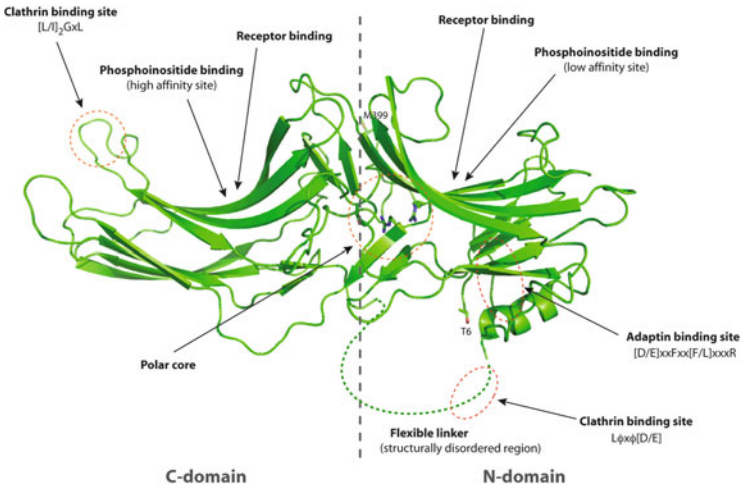

Fig. 2 Secondary structure of β-arrestin-1L. Ribbon diagram of β-arrestin-1L (residues 6–399) indicating the N- and C-domains, the polar core, and binding sites for the GPCR, phosphoinositides (high-affinity site in C-domain and low-affinity site in N-domain), clathrin (Lϕxϕ[D/E] and $[L/I]_2$GxL motifs), and β2-adaptin ([D/E]xxFxx[F/L]xxxR motif)

from the receptor to interact with positively charged Arg169 (in β-arrestin-1), and ultimately causes release of the C-terminal tail from the polar core (Palczewski et al. 1991; Gurevich 1998; Vishnivetskiy et al. 2000; Gurevich and Gurevich 2004). This leads to the disruption of the basal state and subsequent conformational rearrangement of arrestin. Studies monitoring arrestin conformational changes in live cells, along with other biochemical data, suggest that the arrestin amino terminus and C-terminal tail move closer upon binding to an activated receptor (Xiao et al. 2004; Charest et al. 2005). This conformational rearrangement enhances arrestin interaction with receptors and is also thought to expose binding motifs that interact with other proteins such as clathrin and AP2 (Moore et al. 2007).

3 β-Arrestin Interaction with Clathrin

Clathrin is a well-studied endocytic protein that is essential for the formation of clathrin-coated pits (CCPs), which play a central role in receptor endocytosis. Clathrin is composed of a heavy and a light chain and three clathrin molecules associate to form a propeller-shaped triskelion, which is the basic structural unit of CCPs (Kirchhausen 2000). Although most GPCRs internalize via CCPs, GPCRs do not directly bind to clathrin and thus require an adaptor protein to provide a molecular link between the receptor and CCP. While the adaptor protein AP2 plays this role for some GPCRs, β-arrestins also function as adaptors to mediate endocytosis of GPCRs (Ferguson et al. 1996; Goodman et al. 1996). Upon agonist stimulation, β-arrestin-1 was found to colocalize with clathrin and the β_2AR.

Mechanistic studies reveal that β-arrestin-1 and β-arrestin-2 specifically bind to clathrin with a K_d of 10–60 nM (Goodman et al. 1996). The primary clathrin-binding site in β-arrestin, called a clathrin-binding box or Lϕxϕ[D/E] motif (where ϕ is a bulky hydrophobic residue and x represents any polar amino acid), is localized in the carboxyl terminal region (residues 376–380 in β-arrestin-1) (Fig. 2). This motif is also found in many other clathrin-binding proteins such as AP2, AP180, amphiphysin, and epsin (Owen et al. 2004). Importantly, mutation or deletion of this motif in β-arrestin-1 effectively disrupts clathrin binding and receptor internalization (Krupnick et al. 1997; Kim and Benovic 2002; Burtey et al. 2007). Mutagenesis studies localized the β-arrestin binding site to the N-terminal domain of the clathrin heavy chain, specifically residues 89–100, with an invariant Glu89 and conserved Lys96 and Lys98 as critical resides that mediate β-arrestin interaction (Goodman et al. 1997). Hydrophobic and basic residues in this region of clathrin complement the hydrophobic and acidic amino acids within the Lϕxϕ[D/E] motif in β-arrestin.

Crystallographic structures of the terminal domain of the clathrin heavy chain (residues 1–363) in complex with a β-arrestin-2 peptide (ter Haar et al. 2000) as well as with full-length β-arrestin-1 (Kang et al. 2009) support the predicted location of the arrestin–clathrin interface determined by mutagenesis. These structures clearly demonstrate that the Lϕxϕ[D/E] motif in β-arrestin interacts with a hydrophobic patch formed by the first and second blades of the clathrin terminal domain. In addition, charged residues outside of the Lϕxϕ[D/E] motif form hydrogen bonds with Glu89 and Lys96 in clathrin and help to stabilize the interaction. β-arrestin-1 actually exists in two isoforms (long and short) that differ by an eight-amino acid insert between the 18th and 19th β-strands (Sterne-Marr et al. 1993; Kang et al. 2009). Interestingly, the structure of a complex between the long isoform of β-arrestin-1 (β-arrestin-1L) and clathrin revealed a second region of interaction between these proteins. This interaction was mediated by the eight-amino acid insert unique to β-arrestin-1L and a hydrophobic patch formed by fourth and fifth blades of clathrin (Kang et al. 2009) (Fig. 2). Site-directed mutagenesis of the 8-amino acid insert in β-arrestin-1L identified a $[L/I]_2GxL$ motif that mediates clathrin binding. Interestingly, this motif is also found in many other clathrin-binding proteins, although whether it plays a broad role in clathrin binding is currently unknown.

Cell biological approaches have also been used to characterize the functional role of the clathrin-binding motifs in β-arrestin-1L. β-arrestin-1L mutants lacking a single clathrin-binding motif showed reduced β_2AR endocytosis, while β-arrestin-1L lacking both clathrin-binding motifs effectively disrupted clathrin binding and β_2AR endocytosis (Kang et al. 2009). Taken together, these studies demonstrate that β-arrestin interaction with clathrin plays an essential role in endocytosis of many GPCRs, while the two independent interactions between β-arrestin-1L and clathrin likely facilitate the formation of a macromolecular complex that regulates the dynamics of receptor endocytosis.

4 β-Arrestin Interaction with AP2

Another essential component of CCPs is the adaptor protein AP2. AP2 is a heterotetrameric protein consisting of α, β2, μ2, and σ2 subunits and it functions as a clathrin adaptor and in cargo recruitment to CCPs (Owen et al. 2004). The α-adaptin and β2-adaptin subunits of AP2 function in cargo and adaptor recruitment and are composed of ear (appendage), hinge, and trunk domains. The appendage domain of α-adaptin interacts with DP[F/W], FxDxF, and WxxF motifs, while the appendage domain of β2-adaptin interacts with [D/E]xxFxx[F/L]xxxR. The μ2 subunit of AP2 also binds cargo proteins and interacts with Yxxϕ and [D/E]xxL [L/I] motifs as well as with phosphatidylinositol.

Initial studies from the Caron laboratory identified a direct interaction between β-arrestin and β2-adaptin (Laporte et al. 1999, 2000). They found that deletion of 25 amino acids from the C terminus of β-arrestin-1 completely disrupted interaction with β2-adaptin, while mutation of Arg394 or Arg396 in β-arrestin-2 (equivalent to Arg393 and Arg395 in β-arrestin-1) disrupted β2-adaptin binding. Moreover, a β-arrestin-2-R396A mutant did not colocalize with AP2 in CCPs upon receptor activation, in contrast to wild-type β-arrestin-2 (Laporte et al. 2000). Additional studies revealed an essential role for Phe391 and Arg395 in β-arrestin-1 binding to β2-adaptin and showed that F391A and R395E mutants functioned as effective dominant-negative mutants in β_2AR internalization assays when clathrin binding was also disrupted (Kim and Benovic 2002). Several studies also identified the residues in β2-adaptin that mediate β-arrestin binding and revealed an important role for Arg834, Trp841, Glu849, Tyr888, and Glu902 (Kim and Benovic 2002; Edeling et al. 2006; Schmid et al. 2006).

Based on extensive mutagenesis and biochemical analysis, a β2-adaptin-binding consensus sequence was defined as [D/E]xxFxx[F/L]xxxR in β-arrestins, epsin, and autosomal recessive hypercholesterolemia protein (ARH) (Edeling et al. 2006; Schmid et al. 2006). Crystallographic studies demonstrate that the appendage domain of β2-adaptin consists of platform and sandwich subdomains. X-ray structures of β2-adaptin crystallized with synthetic peptides containing the [D/E]xxFxx[F/L]xxxR motif from either ARH (Edeling et al. 2006) or β-arrestin-1 (Schmid et al. 2006) show a molecular interface primarily formed by hydrophobic interactions between the C-terminal domain of β-arrestin-1 and the platform domain of β2-adaptin. The center of this interaction is formed by Phe388 and Phe391 in β-arrestin-1 and Tyr888 in β2-adaptin. Interestingly, the β-arrestin-1 region involved in this interaction forms the last β-strand in holo-β-arrestin-1, while this region becomes α-helical when bound to β2-adaptin, at least when bound as a peptide. These results suggest a conformational change occurs upon arrestin/adaptin binding and support previous findings that arrestin activation promotes adaptin binding (Kim and Benovic 2002). While these studies suggest a major conformational change occurs in β-arrestin when it binds to β2-adaptin, it will be important to validate such results in β-arrestin complexes with receptor and β2-adaptin.

5 β-Arrestin Interaction with Phosphoinositides

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is an important component in clathrin-mediated endocytosis and is mainly enriched at the plasma membrane, although it is also detected in the Golgi, endosomes, and endoplasmic reticulum (Watt et al. 2002). Clathrin-mediated endocytosis can roughly be divided into five stages: nucleation, cargo selection, coat assembly, scission, and uncoating (McMahon and Boucrot 2011). PIP_2 synthesis is important for the nucleation, cargo selection, and coat assembly of CCPs, while scission and uncoating of CCPs are partially dependent on the localized turnover of PIP_2 (Antonescu et al. 2011; Zoncu et al. 2007). GPCR trafficking is also dependent on PIP_2 since alteration of plasma membrane PIP_2 levels significantly affects GPCR endocytosis and recycling (Tóth et al. 2012).

Phosphoinositides also play an important role in β-arrestin-mediated trafficking of GPCRs. β-arrestin-1 and β-arrestin-2 contain a high-affinity phosphoinositide-binding site located in the C-domain where three basic residues (Lys-233, Arg-237, and Lys-251 in β-arrestin-2) have been implicated in phosphoinositide binding (Gaidarov et al. 1999). Mutation of these three residues in β-arrestin-2 (β-arrestin-2-KRK/Q) failed to support β_2AR recruitment to CCPs and subsequent internalization, suggesting that phosphoinositides are important in delivering the receptor–arrestin complex to CCPs. The β-arrestin-2-KRK/Q mutant, however, retains the ability to bind to receptor and clathrin and was recruited to the plasma membrane upon receptor activation (Gaidarov et al. 1999). Various phosphoinositides were found to have the following affinities for β-arrestin-1 and β-arrestin-2: IP_6 (~0.08 μM) > PIP_3 (~0.3 μM) > PIP_2 (~1.4 μM) > IP_4 (~4 μM) > IP_3 (~20 μM) (Gaidarov et al. 1999). Interestingly, β-arrestins also appear to regulate the production of PIP_2 since β-arrestin-2 binds the enzyme phosphatidylinositol 4-phosphate 5-kinase (PIP5K), which functions in PIP_2 production (Nelson et al. 2008). Overexpression of a β-arrestin-2 mutant lacking PIP_2 binding abolishes PIP5K interaction and inhibits β_2AR internalization. A positive feedback mechanism was proposed where β-arrestin-2 interaction with PIP_2 facilitates β_2AR internalization by promoting interaction with PIP5K to synergistically produce more PIP_2, thereby leading to increased local concentrations of PIP_2 (Nelson et al. 2008). Taken together, these results suggest an essential role for phosphoinositides in β-arrestin-mediated trafficking of GPCRs.

While PIP_2 and PIP_3 are the proposed physiological ligands for β-arrestins at the plasma membrane, inositol hexakisphosphate (IP_6), a soluble inositol polyphosphate, displays a higher binding affinity for β-arrestins than either PIP_2 or PIP_3 (Gaidarov et al. 1999). IP_6 is abundant in cells with concentrations ranging between 15 and 100 μM and has been proposed to regulate receptor endocytosis and receptor signaling (Sasakawa et al. 1995). Interestingly, IP_6 inhibits both β-arrestin-1 and β-arrestin-2 binding to an activated phosphorylated GPCR (Gaidarov et al. 1999). Moreover, the ability of IP_6 to bind to two distinct sites on β-arrestins appears to mediate homo- and hetero-oligomerization of β-arrestin-1 and β-arrestin-2 (Milano et al. 2006). Mutation of either IP_6-binding site in β-arrestin-1 disrupted oligomerization, while interactions with known binding partners including clathrin, AP2, and ERK2 were maintained.

Moreover, subcellular localization studies showed that β-arrestin-1 oligomers and β-arrestin-1/2 hetero-oligomers are primarily cytoplasmic, whereas β-arrestin-1 monomers displayed increased nuclear localization (Milano et al. 2006; Storez et al. 2005). This suggests that IP_6 binding to β-arrestins may regulate arrestin localization and function as a negative regulator of arrestin interaction with plasma membrane and nuclear signaling proteins.

6 Additional Interactions Involved in β-Arrestin-Mediated Trafficking of GPCRs

While β-arrestin interactions with clathrin, AP2, and phosphoinositides appear critical in arrestin-promoted endocytosis, β-arrestins also bind several additional proteins that regulate GPCR trafficking (Table 1). For example, β-arrestin-2 interacts with endothelial NO synthase, which promotes S-nitrosylation of β-arrestin-2, which, in turn, enhances association with CCPs and accelerates GPCR internalization (Ozawa et al. 2008). β-arrestin-1 also interacts with *N*-ethylmaleimide-sensitive fusion protein (NSF), an ATPase that regulates intracellular transport. Interestingly, β-arrestin-1 interaction with NSF is ATP dependent and overexpression of NSF enhances agonist-promoted internalization of the β_2AR (McDonald et al. 1999). β-arrestin-1 interaction with Arf6·GDP and its nucleotide exchange factors, ARNO and EFA6, leads to Arf6 activation and subsequent regulation of GPCR endocytosis and recycling (Mukherjee et al. 2000; Claing et al. 2001; Macia et al. 2012). β-arrestins have also been demonstrated to recruit E3 ubiquitin ligases and deubiquitinases to the plasma membrane to regulate GPCR trafficking. For example, β-arrestin-2 is rapidly ubiquitinated by Mdm2 upon β_2AR stimulation and depletion of Mdm2 by siRNA, or overexpression of dominant-negative Mdm2 attenuates β-arrestin-2 ubiquitination and β_2AR internalization (Shenoy et al. 2001, 2009). Moreover, β-arrestin-2 acts as an adaptor between β_2AR and the E3 ubiquitin ligase Nedd4 to facilitate β_2AR ubiquitination and trafficking (Shenoy et al. 2008; Han et al. 2013). Interestingly, β-arrestin-2 can also be modified by sumoylation at Lys-400 and inhibition of β-arrestin-2 sumoylation attenuates β_2AR internalization (Wyatt et al. 2011).

Once internalized, GPCRs are either recycled back to the plasma membrane or sorted to the lysosome and degraded (Fig. 1). The dynamic regulation of GPCR ubiquitination by ubiquitin E3 ligases and deubiquitinases plays a crucial role in endocytic sorting (Marchese and Benovic 2001; Shenoy et al. 2001, 2008, 2009). β-arrestins function to facilitate this process, and the stability of β-arrestin ubiquitination and its interaction with a GPCR appear to contribute to whether a receptor is to be recycled or degraded. For example, agonist stimulation of the β_2AR induces transient ubiquitination and complex formation with β-arrestin-2, and the receptor is rapidly dephosphorylated and recycled after internalization. In contrast, stimulation of the AT_{1a} receptor promotes sustained binding and ubiquitination of β-arrestin-2, and the receptor is effectively sorted to lysosomes and degraded (Oakley et al. 1999, 2001; Shenoy et al. 2001; Shenoy and Lefkowitz 2003). While sustained

Table 1 Protein interactions with β-arrestins that function in GPCR trafficking

Binding partner	Region of the binding partner interacting with arrestin	Region of arrestin interacting with the binding partner	Functions	References
β2-Adaptin	The groove between α-helix 1 and the antiparallel β-sheet of the platform subdomain	[D/E]xxFxx[F/L] xxxR in the C-terminal tail	Directly interacts with β-arrestins and facilitates GPCR endocytosis	Laporte et al. (2000) Edeling et al. (2006) Schmid et al. (2006)
μ2-Adaptin	–	[Y/F]VTL in the N-terminus	Preferentially interacts with β-arrestin-2 and facilitates β_2AR endocytosis	Marion et al. (2007)
Clathrin	Pocket formed by blades 1 and 2 (E89, K96, K98) Hydrophobic pocket formed by blades 4 and 5	LØxØ[D/E] in the C-tail $[L/I]_2$GxL (β-arrestin1L)	Directly interacts with β-arrestins and facilitates GPCR endocytosis	Goodman et al. (1997) ter Haar et al. (2000) Kang et al. (2009)
PIP2	Phosphate head group	Residues 223–285 (K233, R237, K251 in β-arrestin-2)	Directly interacts with β-arrestins and enhances GPCR endocytosis	Gaidarov et al. (1999) Nelson et al. (2008)
PIP5K-Iα	–	Residues 240–261	Directly interacts with β-arrestin-2 and facilitates β_2AR endocytosis	Nelson et al. (2008)
IP6	Phosphate head group	C-domain (K233, R237, K251, K324, K326); N-domain (K157, K160, R161)	Directly interacts with β-arrestins, inhibits β-arrestin/GPCR interaction, facilitates β-arrestin homo- and hetero-oligomerization, and regulates β-arrestin cellular localization	Gaidarov et al. (1999) Storez et al. (2005) Milano et al. (2006)
PI3K	PIK domain	–	Regulates β_2AR endocytosis by AP2 recruitment to the β_2AR/β-arrestin complex	Naga Prasad et al. (2002)
NSF	–	–	Directly interacts with β-arrestin-2 and enhances β_2AR endocytosis	McDonald et al. (1999)

(continued)

Table 1 (continued)

Binding partner	Region of the binding partner interacting with arrestin	Region of arrestin interacting with the binding partner	Functions	References
ARF6	–	C-tail	GDP-bound form interacts with β-arrestin1; enhances GPCR endocytosis; negatively controls recycling; enhances receptor degradation	Claing et al. (2001) Houndolo et al. (2005) Macia et al. (2012)
ARNO	–	–	Activates ARF6 to facilitate β-arrestin release from LH/CGR	Mukherjee et al. (2000)
EFA6	–	C-tail of β-arrestin-1	β-arrestin-1 scaffolds ARF6-GDP and EFA6 to facilitate ARF6 activation leading to β_2AR degradation	Macia et al. (2012)
Mdm2	–	N-domain	Ubiquitinates β-arrestin-2 and facilitates β_2AR endocytosis	Shenoy et al. (2001)
AIP4	WWI–II domains	N-domain (residues 1–260)	Interacts with β-arrestin-2 on early endosomes and facilitates CXCR4 degradation	Bhandari et al. (2007)
Nedd4	–	–	Interacts with β-arrestin to facilitate β_2AR ubiquitination and trafficking	Shenoy et al. (2008) Han et al. (2013)
STAM-1	GAT domain	Residues 25–161	Interacts with β-arrestin-1 to regulate CXCR4 sorting	Malik and Marchese (2010)
USP20	–	–	Directly deubiquitinates β-arrestin-2 and β_2AR to prevent receptor degradation	Shenoy et al. (2009)

(continued)

Table 1 (continued)

Binding partner	Region of the binding partner interacting with arrestin	Region of arrestin interacting with the binding partner	Functions	References
USP33	–	–	Directly deubiquitinates β-arrestin-2 and β_2AR to prevent receptor degradation	Berthouze et al. (2009)
eNOS	–	N terminus	Interacts with and s-nitrosylates β-arrestin-2 and facilitates β-arrestin-2 binding with clathrin and β-adaptin; promotes receptor internalization	Ozawa et al. (2008)

ubiquitination of GPCRs is important for receptor degradation, deubiquitination of GPCRs regulates receptor recycling back to the plasma membrane. For example, the deubiquitinases USP33 and USP20 have been shown to directly interact with β-arrestin-2 and facilitate both β-arrestin-2 and β_2AR deubiquitination. Importantly, a double knockdown of USP20 and USP33 enhances the extent of β-arrestin-2 ubiquitination and increases β_2AR degradation (Berthouze et al. 2009; Shenoy et al. 2009).

Once a GPCR is committed to the degradation pathway, it is sorted to the lysosome with the help of the ESCRT complexes (Marchese and Trejo 2013). The presence of a functional β-arrestin has been shown to be essential for effective sorting and degradation of CXCR4. Specifically, β-arrestin-1 colocalizes with atrophin-interacting protein 4 (AIP4), an E3 ubiquitin ligase, on early endosomes to facilitate CXCR4 sorting and degradation. Knockdown of β-arrestin-1 inhibits CXCR4 degradation but does not affect CXCR4 ubiquitination or internalization (Bhandari et al. 2007). CXCR4 sorting is also regulated by β-arrestin-1 interaction with signal-transducing adaptor molecule-1 (STAM-1), and disruption of this interaction attenuates agonist-promoted ubiquitination of HRS and enhances sorting to lysosomes (Malik and Marchese 2010).

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Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications

Sudha K. Shenoy

Contents

Abstract Arrestins constitute a small family of four homologous adaptor proteins (arrestins 1–4), which were originally identified as inhibitors of signal transduction elicited by the seven-transmembrane G protein-coupled receptors. Currently arrestins (especially arrestin2 and arrestin3; also called β-arrestin1 and β-arrestin2) are known to be activators of cell signaling and modulators of endocytic trafficking. Arrestins mediate these effects by binding to not only diverse cell-surface receptors but also by associating with a variety of critical signaling molecules in different intracellular compartments. Thus, the functions of arrestins are multifaceted and demand interactions with a host of proteins and require an array of selective conformations. Furthermore, receptor ligands that specifically induce signaling via arrestins are being discovered and their physiological roles are emerging. Recent evidence suggests that the activity of arrestin is regulated in space and time by virtue of its dynamic association with specific enzymes of the

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ubiquitination pathway. Ubiquitin-dependent, arrestin-mediated signaling could serve as a potential platform for developing novel therapeutic strategies to target transmembrane signaling and physiological responses.

Keywords Ubiquitin • Beta-arrestin • G protein coupled receptor • Endocytosis • Deubiquitinase • Lysosomes

1 Introduction

Arrestins are multifunctional adaptor proteins that were originally discovered as molecules that uncouple heterotrimeric G proteins from activated G protein-coupled receptors (GPCRs, also known as seven-transmembrane receptors or 7TMRs) (Lefkowitz and Shenoy 2005; Luttrell and Lefkowitz 2002). The arrestin gene family contains four members divided into two subtypes: (1) visual arrestins, namely arrestin1 and arrestin4 that are mostly expressed in retinal rods and cones and (2) nonvisual arrestins, namely arrestin2 (also called β-arrestin1) and arrestin3 (also called β-arrestin2) that are expressed in all mammalian cells (Gurevich and Gurevich 2006). Arrestins associate with membrane-bound receptors that have been agonist stimulated and phosphorylated on serines and threonines by GPCR kinases (GRKs). GRK phosphorylation and arrestin binding thus constitute a two-step process for uncoupling G proteins and desensitizing GPCRs. There are 7 GRKs in mammalian cells; of these GRK1 and GRK7 phosphorylate GPCRs of the visual system, whereas GRKs 2–6 regulate the vast majority of nearly 800 members of the GPCR family.

In addition to receptor desensitization, arrestins also function as critical endocytic adaptors to facilitate GPCR internalization (Ferguson 2001; Moore et al. 2007). β-arrestins bind clathrin, which is a structural component of endocytic vesicle and adaptin protein 2, which functions together with clathrin to transport specific cargo through the endocytic pathway (Claing et al. 2002; Mishra et al. 2005; Moore et al. 2007). The endocytic adaptor function of β-arrestin is continually expanding to include new interacting proteins of the endocytic machinery and other families of cell-surface receptors, thus underscoring their importance in endocytic trafficking (Shenoy and Lefkowitz 2011; Shukla et al. 2011). Through the second decade after their cloning, β-arrestins were shown to function as signaling adaptors for the non-receptor tyrosine kinase c-Src, extracellular signal regulated kinases 1 and 2 (ERK1/2), c-Jun-N-terminal kinase 3 (JNK3), p38, and AKT (Beaulieu et al. 2009; DeWire et al. 2007). GPCR ligands that specifically activate β-arrestin-mediated signaling have also been identified (DeWire et al. 2008; Luttrell and Kenakin 2011; Reiter et al. 2012; Urban et al. 2007). These studies provide a growing impetus for therapeutic targeting of selected signaling pathways evoked by GPCRs: G protein versus β-arrestin dependent (Violin et al. 2013; Whalen et al. 2011).

Recent studies have revealed that a phosphorylation barcode conferred by individual GRK isoforms is correlated with the activation of β-arrestin to a specific conformation and is integrated with specific downstream effects (Busillo et al. 2010; Nobles et al. 2011; Zheng et al. 2012; Zidar et al. 2009). For example, GRK2 sites on the β_2 adrenergic receptor (β_2AR) direct the conformation for desensitization and endocytosis, whereas GRK6 sites engage a β-arrestin conformation to propagate MAP Kinase signaling (Nobles et al. 2011) (see Chaps. 12–14). While the functions and protein interactions of β-arrestins are continually expanding, molecular mechanisms that regulate these diverse roles are not fully understood. β-Arrestins undergo post-translational modifications which correlate with their activation and these molecular changes in β-arrestin conformation affect their protein interactions leading to modulation of receptor endocytosis and signaling (Shenoy 2007; Shenoy and Lefkowitz 2011). The ubiquitous and reversible protein post-translational modification called ubiquitination [for a detailed review, see reference (Hershko and Ciechanover 1998)] in which the protein ubiquitin becomes covalently appended to substrate proteins by E3 ubiquitin ligases and removed by deubiquitinases (DUBs) plays a critical role in endocytic and signaling pathways transduced by 7TMR–β-arrestin complexes (Shenoy 2007; Shenoy et al. 2007, 2009).

The interaction of β-arrestins with the enzymatic machinery that controls protein ubiquitination serves as a checkpoint for fine-tuning activities of both β-arrestins and the proteins that they interact with. Additionally, a balance between ubiquitin conjugation and deconjugation governs the efficiency of recycling of internalized receptors versus their degradation in the lysosomes (Berthouze et al. 2009; Shenoy and Lefkowitz 2011; Shenoy et al. 2008). Thus, specific nodes in the ubiquitin pathway can be exploited for therapeutic targeting of β-arrestin-dependent trafficking and signaling.

2 Arrestin–E3 Ubiquitin Ligase Interaction

E3 ubiquitin ligases are enzymes that catalyze the final step of the enzyme cascade that appends ubiquitin to lysine residue(s) of substrate proteins. E3 ligases transfer the ubiquitin moiety that is initially activated by E1 (ubiquitin-activating enzyme) and then carried by E2 (ubiquitin-carrying enzyme) (Hershko and Ciechanover 1998). Human cells have two E1s, ~60 E2, but more than 600 E3 enzymes, and hence substrate specificity is mostly dictated by E3 interaction (Metzger et al. 2012). E3 ligases are mainly categorized into two types, namely, HECT (homologous to E6AP C terminus) and RING (really interesting new gene) family ligases based on the structure of the catalytic domains. E3 ligases can directly interact with substrates through recognition domains or by cues resulting from other post-translational modifications; however, E3 ligases often connect to substrates via an adaptor protein (Becuwe et al. 2012).

Substrate ubiquitination was originally defined in the context of non-lysosomal degradation of cytoplasmic proteins, which is carried out by the multi-subunit protease complexes called 26S proteasomes (Hershko and Ciechanover 1998). Because ubiquitin itself has seven internal lysines as well as a free amino terminus, all of which can serve as acceptor sites for successive rounds of ubiquitin attachment, the net result is the formation of a polyubiquitin chain on the substrate. Thus ubiquitination can embellish a protein in many ways: with a single ubiquitin on a single lysine (monoubiquitination), single ubiquitin on many individual lysines (multi-monoubiquitination), homogenous polyubiquitin chains at a particular lysine in ubiquitin, mixed chain linkages on ubiquitin, or a combination of any of the above patterns. Accordingly, ubiquitination presents a complete array of chain topologies and a major alteration of the tertiary conformation of the substrate protein. Additionally, each type of ubiquitination pattern could serve as a tag or a code for a particular function or fate: lysine48-linked ubiquitin chains promote proteasome-mediated destruction, whereas lysine63-linked chains tag proteins for endocytosis, activation of kinases, or protein interactions (Chen 2012; Mukhopadhyay and Riezman 2007). As described below, different E3 ligases have been shown to associate with β-arrestins, and, in specific cases, this interaction results in β-arrestin ubiquitination, whereas in many instances, β-arrestin functions as an adaptor to escort the E3 ligase to either a receptor or to an interacting protein.

2.1 Arrestin as a Substrate

The RING domain-containing protein Mdm2 was the first E3 ubiquitin ligase shown to interact with β-arrestin and was identified as a β-arrestin-binding protein in yeast two-hybrid screens (Shenoy et al. 2001). Mdm2 specifically ubiquitinates β-arrestin2 upon β_2AR stimulation and the pattern of ubiquitination is transient. Additionally, such ubiquitination of β-arrestin2 leads to non-degradative effects. Blocking Mdm2-dependent ubiquitination of β-arrestin2 impairs the ability of β-arrestin to augment receptor endocytosis and this is attributed to a decrease in association between β-arrestin and clathrin as well as a decrease in β-arrestin–receptor interaction when β-arrestin is not ubiquitinated (Shenoy et al. 2001, 2007, 2008, 2009). Additionally, ubiquitination of β-arrestin2 is also critical for its MAP Kinase scaffolding: siRNA-mediated knockdown of Mdm2 in HEK-293 cells diminished β-arrestin-dependent ERK activation, whereas exogenous Mdm2 augmented it (Shenoy et al. 2009). These findings are also supported by parallel studies that were conducted with modified β-arrestins (a) containing partial or complete elimination of lysines such that ubiquitination is impaired or eliminated and (b) fusion of ubiquitin in frame so that ubiquitination is persistent (Shenoy et al. 2007; Shenoy and Lefkowitz 2005). Accordingly, ubiquitin tags on β-arrestin facilitate tight interaction with activated 7TMRs, as well as with endocytic protein partners and further govern the assembly and localization MAP Kinase scaffolds on endosomes or signalsomes. In contrast, elimination of ubiquitination impairs each of these functions and interactions of β-arrestin (Fig. 1).

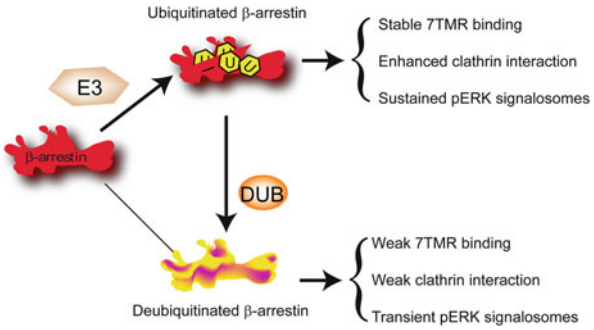

Fig. 1 Reciprocal regulation of β-arrestin functions by E3 ligases and deubiquitinases. E3 ubiquitin ligases such as Mdm2 increase and stabilize β-arrestin ubiquitination in response to agonist activation of GPCRs (here reference is made to the β_2AR). The ubiquitinated β-arrestin is empowered to stably interact with the receptor, clathrin, and scaffold MAP Kinase activity on signaling endosomes. Deubiquitinases such as USP33 promote deubiquitination of β-arrestin in response to β_2AR activation and destabilize the interaction with the receptor and clathrin and do not scaffold MAP Kinase activity on endosomes. Unmodified β-arrestin in the basal state may or may not have the same conformation as the de-ubiquitinated β-arrestin

Ubiquitination of β-arrestin is conformation specific and it is likely that distinct conformations of β-arrestins induced by different GPCRs will engage different E3 ligases. Additionally the site of modification on β-arrestins can also be specific for a receptor–β-arrestin pair (Shenoy and Lefkowitz 2005). While Mdm2 serves as the obligatory E3 ligase for β_2AR-stimulated ubiquitination of β-arrestin2, it may not be the specific E3 that modifies β-arrestin2 associated with the V_2 vasopressin receptor (V_2R) tail because in these two cases, the conformation of activated β-arrestin2 is different (Shenoy et al. 2009). In addition to Mdm2, other E3 ubiquitin ligases, namely, Nedd4 (Shenoy et al. 2008), AIP4 (Bhandari et al. 2007), and Parkin (Ahmed et al. 2011) have also been shown to bind β-arrestin; however, these interactions do not seem to result in β-arrestin ubiquitination. β-Arrestin has also been shown to be SUMOylated upon β_2AR stimulation: SUMO or small ubiquitin-like modifier is a ubiquitin-like protein that is also appended to lysines in substrate proteins (Johnson 2004; Wyatt et al. 2011). Arrestin SUMOylation has been mapped to a lysine residue unique to bovine arrestin3 and attenuation of this modification by an unknown SUMO-E3 ligase reduces the association between arrestin3 and β-adaptin2. Although this functional SUMO site is absent in other β-arrestin orthologs, there are other conserved SUMO motifs in arrestins and GRKs (Shenoy and Lefkowitz 2011). Currently the interdependence or antagonism between ubiquitination and SUMOylation on arrestins and the corresponding effects on signaling are unknown.

2.2 Arrestin as an Adaptor in Ubiquitination

The E3 ubiquitin ligases or DUBs recognize their substrates via specific protein interaction domains or conformational cues appended on the substrate by another post-translation modification (e.g., phosphorylation/dephosphorylation) (Hershko and Ciechanover 1998). More often adaptor molecules function as accessory proteins to escort E3 ubiquitin ligases or scaffold both E2 and E3 enzymes (Hershko and Ciechanover 1998; Leon and Haguenauer-Tsapis 2009; Shenoy and Lefkowitz 2011). Collectively various mechanisms are in place to regulate the fidelity, timing, localization, and extent of substrate ubiquitination.

Both β-arrestin isoforms function as critical E3 ligase adaptors for GPCRs, other cell surface receptors, and non-receptor proteins to mediate ubiquitination. β-Arrestin2 functions as a required adaptor for the E3 ligase Nedd4 to mediate ubiquitination of the agonist-activated β_2AR (Han et al. 2013; Shenoy et al. 2008). β-Arrestins recruited to the activated β_2ARs show sequential interactions with two E3s: first with Mdm2 which ubiquitinates β-arrestin2 followed by Nedd4 that ubiquitinates the β_2AR (Shenoy et al. 2008). This can be explained by the kinetics of agonist-induced association of these molecules. While Mdm2-β-arrestin binding increases immediately (up to 5 min) after agonist stimulation, it decreases at later time points (>15 min). On the other hand, the peak timing for Nedd4–β-arrestin association is 5–15 min after agonist stimulation (Shenoy et al. 2008). Nedd4 and other HECT-domain ligases possess WW domains that act as binding platforms for polyproline motifs on substrates or adaptors (Ingham et al. 2005). However, β-arrestin2 binds to Nedd4 even when all the WW domains are mutated suggesting a WW-independent interaction (Shenoy et al. 2008). Interestingly Nedd4 promotes trafficking of activated β_2ARs into endosomes that contain a secondary endosome-localized arrestin-domain-containing adaptor (ARRDC2, ARRDC3, or ARRDC4) and an ESCRT-0 protein called Hrs1 (Han et al. 2013). ARRDCs bear little sequence identity with arrestins, but are predicted to fold into an arrestin-like structure. They also contain polyproline motifs that bind to the WW domains in Nedd4 and other HECT-domain E3s. Mutation of the polyproline motifs in ARRDC 2, 3, and 4 eliminates their interaction with Nedd4 and endosomal β_2ARs and blocks their localization on endosomes (Han et al. 2013). ESCRTs are protein complexes that recognize ubiquitinated cargo on vesicles and direct their intracellular trafficking to late endosomes or multivesicular bodies, which subsequently fuse with the lysosomes (Katzmann et al. 2001). The functional role of β_2AR ubiquitination is distinct from that of β-arrestin ubiquitination. Ubiquitination of the β_2AR tags internalized receptors for lysosomal degradation (Shenoy et al. 2001). For the β_2AR, receptor ubiquitination in either the third intracellular domain or the carboxyl tail is required for lysosomal trafficking and degradation (Xiao and Shenoy 2011).

The adaptor role for β-arrestin has also been reported for ubiquitination of the V_2R and μ-opioid receptor, but the identity of the E3 ligases is not yet known (Groer et al. 2011; Martin et al. 2003). A growing list of 7TMRs is shown to be

ubiquitinated, yet there is a lag with regard to the assignment of a role for β-arrestins (Alonso and Friedman 2013; Decaillot et al. 2008; Dores and Trejo 2012). On the other hand, there are examples of β-arrestin-independent ubiquitination. β-Arrestin is not required for AIP4-mediated ubiquitination of the chemokine receptor CXCR4 (Bhandari et al. 2007). Another interesting scenario is with the β_2AR, when it is bound by the β-blocker carvedilol. Although carvedilol-stimulated MAP Kinase signaling is mediated by recruited β-arrestins (Wisler et al. 2007), the subsequent ubiquitination and lysosomal trafficking of carvedilol-bound β_2ARs are independent of both β-arrestin2 and Nedd4, and dependent on a PHD-domain E3 ligase called MARCH2 (Han et al. 2012). Additionally, while the agonist-induced ubiquitination is targeted to specific lysines in the β_2AR, carvedilol-induced ubiquitination also involves non-canonical sites (cysteines and serines) (Han et al. 2012; Xiao and Shenoy 2011). Thus, the agonist isoproterenol and the antagonist (or a weak β-arrestin-biased agonist) carvedilol, display selective engagement of distinct E3 ligase components and target different ubiquitin acceptor sites to effect β_2AR trafficking. Moreover, while agonist-dependent internalization of the β_2AR proceeds in the absence of Nedd4-mediated ubiquitination, carvedilol-induced internalization requires MARCH2-mediated ubiquitination of the β_2AR (Han et al. 2012). These findings suggest that the ubiquitin tag(s) on a 7TMR can serve distinct functions in conjunction with the specific ligand-bound conformation of the receptor.

β-Arrestins have also been shown to regulate ubiquitination of diverse receptors and transmembrane proteins as well as a growing list of kinases and other signaling proteins (Shenoy and Lefkowitz 2011). In addition to the β_2AR, β-arrestins can escort HECT-domain E3 ligases to ubiquitinate other plasma membrane proteins: AIP4 for the transient receptor potential (TRP) ion channel family member TRPV4; Nedd4-1 for the Na^+/H^+ exchanger 1 (NHE1); and Drosophila Deltex, which is recruited by the fly arrestin called Kurtz to ubiquitinate and regulate the Notch receptor (Hori et al. 2011; Shukla et al. 2010; Simonin and Fuster 2010). The β-arrestin2–Mdm2 adaptor–E3 complex ubiquitinates and regulates androgen receptor levels in prostate cancer cells (Lakshmikanthan et al. 2009). β-Arrestin1 functions as an essential adaptor to escort Mdm2 and mediate ubiquitin-dependent proteasomal degradation of insulin-like growth factor 1 receptors (IGF-1Rs) and the tumor suppressor protein p53 (Girnita et al. 2005; Hara et al. 2011). The latter is triggered by chronic β_2AR activation and leads to impaired apoptosis and accumulation of DNA damage. Mdm2 functions as an E3 ligase for at least two more regulatory enzymes upon βAR activation (1) for the ubiquitin-dependent degradation of GRK2, which requires one of the β-arrestin isoforms in addition to c-Src-mediated tyrosyl phosphorylation of GRK2 (Salcedo et al. 2006) and (2) for monoubiquitination of PDE4D5, a cAMP-metabolizing enzyme that is scaffolded by β-arrestin2, where the ubiquitin constitutes a protein–protein interaction tag to differentiate scaffolding by β-arrestin2 versus by RACK1 (Li et al. 2009).

3 Arrestin–DUB Interaction

Ubiquitinated proteins are deubiquitinated by specific proteases known as deubiquitinases (DUBs, also known as ubiquitin-specific proteases or USPs) and these proteases cleave the isopeptide bond between carboxyl terminus of ubiquitin and the amino group of the substrate (Reyes-Turcu et al. 2009). Bioinformatics analyses have identified that the human genome encodes ~100 putative DUBs, of which 79 are suggested to be functional. The DUBs have been divided into five distinct classes based on sequence and structural homology (Reyes-Turcu et al. 2009). Four of the five classes identified to date are cysteine proteases with a classical papain active catalytic site structure encompassing the catalytic triad of cysteine, histidine, and a third residue, which is aspartic acid, asparagine, or rarely serine. These four classes are (1) ubiquitin carboxy-terminal hydrolases (UCH; 4 members); (2) ubiquitin-specific proteases (USP; over 58 members); (3) Machado–Joseph Domain (MJD; 5 members), and (4) ovarian tumor-related (OUT) family (14 members). In contrast, the fifth class called jab1/MPN domain-associated metalloisopeptidase (JAMM) motif DUBs are Zn^{2+}-containing metalloproteases (14 members). Studies have demonstrated that DUBs play an important role in several aspects of the ubiquitin–proteasome system and mutations in several DUBs have been implicated in a number of diseases ranging from hereditary cancer to neurodegeneration. Because DUB family members possess a variable domain architecture flanking their conserved catalytic domain, a significant degree of substrate specificity is expected. Although findings about DUB specificity for substrates as well as types of ubiquitin chains that they act upon are continually emerging, our knowledge about the DUB family is far from complete. Nonetheless, the involvement of these enzymes in many regulatory steps in cell physiology and their dysregulation in various diseases portrays them as attractive therapeutic targets.

Initial studies on β-arrestin ubiquitination showed a striking correlation between the kinetics of β-arrestin deubiquitination and the intracellular trafficking of arrestin–receptor complexes (Shenoy and Lefkowitz 2003). GPCRs that formed a stable complex with β-arrestin induced sustained ubiquitination of β-arrestin. On the other hand, GPCRs that formed transient complexes with β-arrestin induced transient pattern of ubiquitination (Dalrymple et al. 2011; Perroy et al. 2004; Shenoy and Lefkowitz 2003). Furthermore, a β-arrestin2–Ub chimera displayed a stable interaction with GPCRs that induced only transient recruitment of wild-type β-arrestin2. The correlation of the kinetics of β-arrestin deubiquitination with the pattern and timing of dissociation of β-arrestins from activated GPCRs suggested a critical role for deubiquitinases in this process. The identification of USP33 as a β-arrestin-interacting protein in a yeast two-hybrid screen confirmed this initial assumption (Shenoy et al. 2009)

Upon USP33 overexpression, V_2R-stimulated effects, namely, stable β-arrestin binding, sustained β-arrestin2 ubiquitination, and persistent ERK activation were all inhibited. On the other hand, targeted gene silencing of USP33 promoted stable

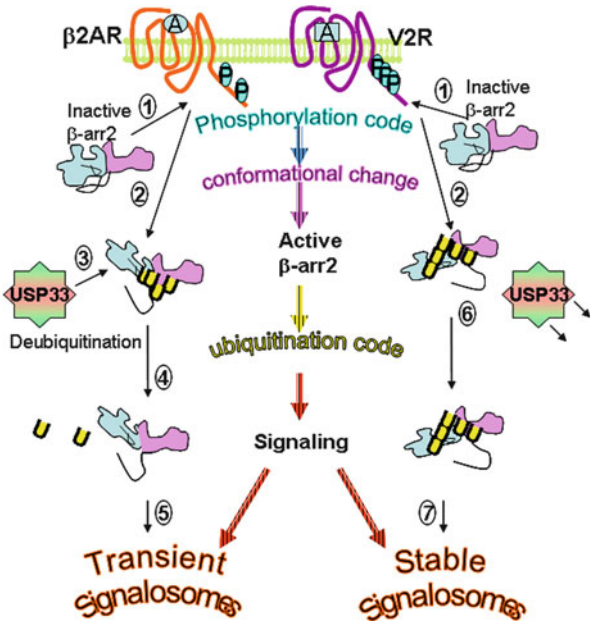

Fig. 2 Effects of post-translational modifications in 7TMR signaling. (1) β-Arrestin2 resides in a basal state in the cytoplasm and is recruited to the plasma membrane and binds phosphorylated C-termini of 7TMRs. The sites of phosphorylation differ among the two representative receptors shown; (2) Upon binding to each receptor, β-arrestin2 undergoes a distinct conformational reorientation, thus allowing distinct regions to become modified by ubiquitination; (3) The β_2AR induced conformation promotes β-arrestin2–USP33 interaction; (4) USP33 deubiquitinates β-arrestin leading to the dissociation of β-arrestin from the β_2AR; (5) β_2AR–β-arrestin2 signalosomes are short lived and promote transient ERK activity that is predominantly non-endosomal; (6) β-arrestin2 conformation induced by V_2R activation prevents USP33 binding, thus protecting β-arrestin ubiquitination, allowing tight binding to activated receptors; (7) V_2R–β-arrestin2 signalosomes are stable and result in robust ERK activity that is predominantly localized on endosomes [Figure and legend adopted from Shenoy et al. (2009)]

interaction and co-internalization of β-arrestin2 and the β_2AR, sustained ubiquitination of β-arrestin2, and prolonged activation of ERK (Shenoy et al. 2009). Accordingly, the kinetics of β-arrestin ubiquitination and deubiquitination are tightly regulated by USP33 ensuring the appropriate duration and magnitude of β-arrestin-biased signaling. USP33 thus functions as an endogenous inhibitor of β-arrestin-dependent signaling provoked by the β_2AR. At a molecular level, these effects are dependent upon conformation-specific interaction of USP33 and β-arrestin2 because β_2AR-induced conformation of "active" β-arrestin2 enhances its binding with USP33, whereas V_2R-induced conformation reduces it (Fig. 2). Accordingly, β-arrestin–receptor interaction is regulated by the phosphorylation motifs on the carboxyl tail of 7TMRs, which is followed by ubiquitination of specific residues by distinct E3 ubiquitin ligase(s) and recruitment and conformation-specific interaction of deubiquitinating enzymes. Therefore, two

types of unique codes dictate β-arrestin-dependent cellular effects: "phosphorylation code" at the receptors generated by specific GRK-mediated phosphorylation followed by "ubiquitination code" on β-arrestin conferred by E3 ubiquitin ligase (s) and perhaps edited by DUBs (Fig. 2).

Interestingly, GPCRs are also regulated by DUBs which reverse ubiquitination and alter the trafficking itinerary of intracellular receptor complexes (Shenoy and Lefkowitz 2011). While agonist stimulation of the β_2AR leads to ubiquitination and lysosomal degradation of the receptor, overexpression of USP33 or USP20 counteracts these effects and promotes receptor recycling and resensitization (Berthouze et al. 2009). Additionally, knockdown of both USP33 and USP20 abolishes receptor recycling and resensitization but enhances ubiquitination as well as lysosomal degradation. USP20 and USP33 thus act as novel regulators that dictate the post-endocytic fate of internalized β_2ARs. These deubiquitinases are constitutively bound to the cell-surface β_2ARs; however, β_2AR–USP association decreases upon agonist activation, while simultaneously, agonist stimulation leads to the recruitment of β-arrestins to the β_2AR with a resultant increase in β-arrestin–USP33 binding. Thus, iso-stimulation induces a reciprocal pattern of USP33 interaction with the β_2AR and β-arrestin2: dissociation of USP33 from the β_2AR and association of USP33 with β-arrestin2 (Berthouze et al. 2009). These data support the idea that while β-arrestin2 facilitates β_2AR ubiquitination by recruiting Nedd4, its adaptor function might actually serve to remove the deubiquitinases from the activated β_2AR to facilitate receptor ubiquitination. It is tempting to speculate that β-arrestin–DUB heterodimers and E3-ligase:β-arrestin:DUB scaffolds will have additional novel roles in orchestrating signal transduction.

4 Therapeutic Possibilities

With the intersection of β-arrestin-dependent pathways and the ubiquitin system, there are numerous routes to therapeutics as well as a host of unique challenges. The magnitude and subcellular localization of β-arrestin-dependent signaling is correlated with its ubiquitination status. β-Arrestin-dependent signaling can be beneficial in certain contexts: for example, averting cardiomyocyte apoptosis during catecholamine-induced stress, promoting anabolic bone formation, etc. (Gesty-Palmer et al. 2009; Noma et al. 2007; Rajagopal et al. 2010). On the other hand, β-arrestin-dependent pathways could have disadvantages as in developing morphine-induced tolerance, constipation, and respiratory depression (Groer et al. 2007). Therefore, in order to target these diverse effects, it would be advantageous to develop inhibitors of E3's that ubiquitinate β-arrestin (to block β-arrestin ubiquitination and signaling) as well as inhibitors of DUBs (to stabilize or augment β-arrestin ubiquitination and signaling).

The druggability of the ubiquitin proteasome system became evident from the development of the proteasome inhibitor Bortezomib (PS-341, Velcade), which is now used for treating multiple cell myeloma and mantle cell lymphoma (Hideshima

et al. 2001; Teicher et al. 1999). Bortezomib is a dipeptide boronic acid analog that binds and inhibits the catalytic site of the proteasome and blocks cell signaling (mainly the NFκB pathway), thus inhibiting cell survival and tumor growth. Because substrates for the proteasome are defined by the balance between the reciprocal activities of E3 ligases and DUBs, considerable efforts have been devoted to developing inhibitors of these enzymes (Eldridge and O'Brien 2010; Nicholson et al. 2007, 2008). The three-dimensional structures of several E3 ubiquitin ligases and DUBs are available (Huang et al. 1999; Ogunjimi et al. 2005; Reyes-Turcu et al. 2009; Verdecia et al. 2003), which points to structure-based design of E3 or DUB inhibitors as a plausible route to therapeutics.

The HECT domain ligases are ideal candidates to seek out an inhibitory small molecule that occupies the catalytic cleft and blocks transfer of activated ubiquitin from the cognate E2. The main difficulty, however, is the fact that available structures are actually snapshots of many potential conformations of the HECT domain, which these E3 enzymes adopt during ubiquitin transfer. These intermediary conformations are possible because of the two mobile structural lobes of the HECT domain (N-lobe and C-lobe) connected by a flexible hinge region; introduction of rigidity within the hinge region by proline mutations diminishes E3 ligase activity (Verdecia et al. 2003). This raises the exciting possibility of developing noncompetitive allosteric inhibitors that block these conformational transitions of HECT domain ligases.

The E3 ligases of the RING domain family pose a different set of challenges. First it is generally accepted that these E3 ligases are not bona fide enzymes; they do not form a covalent bond and accept the activated ubiquitin, but rather position the E2 enzyme and substrate to favor efficient ubiquitin transfer. Conversely, there are numerous reports where mutating the RING domain leads to a loss of substrate ubiquitination, which could be attributed to a different binding mode with the substrate or due to inherent loss of enzymatic activity (Dang et al. 2002; Fang et al. 2000; Jackson et al. 2000; Joazeiro and Weissman 2000). However, unlike the proteasomal inhibitor Velcade, the compounds identified through functional assays based on inhibiting E3 auto-ubiquitination performed for single subunit RING domain E3 ligases have not progressed to clinical trials (Davydov et al. 2004; Lai et al. 2002; Yang et al. 2005). These E3 screens are also confounded by the number of components required to formulate a screening assay (which would minimally require the three enzymes: E1, E2, and E3). Although some compounds with low efficacy or specificity have been identified, they turned out to be either generic for E2 or capable of inhibiting different E3s.

A second set of hurdles is with regard to the subfamily that includes multi-subunit RING E3s. Here the substrate scaffolding is carried out by the Cullin family of proteins and the ubiquitin transfer is associated with distinct RING domain-containing subunits that are held together by a substrate-specific adaptor (which would be one of ~70 F-box proteins) (Deshaies 1999). The multi-subunit nature of these E3 ligases facilitates innumerable combinations of individual subunits involved, which come together to form a functional E3 complex. Therefore, blocking one E3 activity can affect numerous adaptors and in turn block

ubiquitination of multiple substrates each adaptor associates with, thus resonating the effects through multitude of pathways causing overall cell toxicity. In general, the RING domain ligase may require a strategy different from that of attacking the enzymatic activity, one that would interfere with its binding to specific substrates. In fact nutlin, one of the best characterized inhibitors of Mdm2, is a small molecule that binds Mdm2 and prevents its interaction with p53, thus blocking ubiquitination and degradation of p53, which retards tumor growth (Vassilev 2007). A variant of Nutlin is now undergoing clinical evaluation (Shangary and Wang 2009). A similar protein interaction inhibitor that binds p53 called RITA, which showed substantial p53-dependent antitumor effect in vivo, has also been reported (Issaeva et al. 2004). Other antagonists targeting Mdm2–p53 interaction have also been described, some of which are reported to have effects on other Mdm2-binding partners in addition to p53 (Grasberger et al. 2005). Collectively these discoveries indicate that the identification of inhibitors that target protein–protein interaction is a tractable approach. Furthermore, this approach has yielded more therapeutic avenues than the traditional methods of targeting the catalytic activity of RING E3 ligases.

Putting the above advances in therapeutic targeting of the components of the ubiquitin system in the context of β-arrestin and GPCR signaling, one could presume that the complexity of the two networks would make the identification of a useful drug an unrealizable goal. However, the success with the antagonists developed for Mdm2–p53-binding interface support the possibility of finding a similar antagonist(s) for arrestin–E3 ligase or arrestin–DUB-binding interface. So far a three-dimensional structure of arrestin complexed with any of the components of ubiquitin system has not been solved, although individual structures of arrestins, E3s, and DUBs have been described at atomic level (Gurevich and Gurevich 2006; Metzger et al. 2012; Reyes-Turcu et al. 2009). The availability of three-dimensional structure of arrestin–Mdm2 complex would be critical for developing inhibitors of β-arrestin ubiquitination. This would allow tuning down of β-arrestin-dependent pathways and perhaps stabilizing G protein-dependent pathways. Indeed, such a scenario would be preferred to alleviate symptoms of tachyphylaxis during treatment of diseases such as asthma (Whalen et al. 2011). Additionally a reciprocal approach to stabilize ubiquitination of β-arrestin would also be desired and this would require the identification of an inhibitor of USP33 for the β_2AR pathway or a cognate DUB for other GPCR systems. This would allow tuning up of β-arrestin-mediated signaling, which has been found to be beneficial in various physiological contexts, and has been targeted for treating heart failure (DeWire and Violin 2011). With an appropriate screening assay, one could also identify GPCR ligands that promote or prevent specific interactions of β-arrestins with an E3 ligase or a DUB. Finally, a wealth of information has been generated with a variety of arrestin mutants with respect to their cellular activity and GPCR interaction; re-expression of precisely engineered mutant forms of arrestin that is impaired or augmented in ubiquitination would also be an excellent approach to therapeutically target arrestins and their signaling (Gurevich and Gurevich 2010; Gurevich et al. 2008) (see Chap. 1).

5 Conclusions

With our increasing appreciation of the novel and multifaceted functions of arrestins, we are also encountered with the perplexing question as to how these adaptors can interact with so many proteins with specificity and in a timely fashion. The reversible and dynamic nature of ubiquitination of arrestins could be one mode by which they can carry out these multiple interactions: by adorning different shapes and acquiring new "binding surfaces" from the extra ubiquitin moieties on specific regions of the protein. Additionally, some of the resulting interactions might mask or unmask other regions to facilitate the subsequent protein interaction involving arrestins and might promote localization of arrestin complexes to distinct subcellular compartments.

GPCRs are major targets for drug discovery and about 40 % of prescription medications are either agonists or antagonists acting directly or indirectly on these receptors. In addition to therapeutic targeting with GPCR ligands, one should also consider fine tuning the activity of arrestins which occurs downstream of GPCR activation. In this context, use of specific mutants and/or targeted inhibitors of arrestins themselves or approaches to modulate the interacting E3 ubiquitin ligases and DUBs would provide several advantages: first this would preserve the balance of GPCR signal transduction occurring acutely; second it would favor channeling of arrestin signaling through a desired set of signaling nodes; third, it would still preserve GPCR interactions with other proteins that are also crucial for maintaining cellular homeostatsis; and fourth, although speculative, it might be possible to tune a desired function of arrestin as needed in the context of a disease. For example, one could target the initial signaling or protein interaction of arrestin in cell migration and develop novel therapeutic strategies to block cancer metastasis.

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Self-Association of Arrestin Family Members

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Contents

Abstract Mammals express four arrestin subtypes, three of which have been shown to self-associate. Cone photoreceptor-specific arrestin-4 is the only one that is a constitutive monomer. Visual arrestin-1 forms tetramers both in crystal and in solution, but the shape of its physiologically relevant solution tetramer is very different from that in the crystal. The biological role of the self-association of arrestin-1, expressed at very high levels in rod and cone photoreceptors, appears to be protective, reducing the concentration of cytotoxic monomers. The two nonvisual arrestin subtypes are highly homologous, and self-association of both is facilitated by IP6, yet they form dramatically different oligomers. Arrestin-2 apparently self-associates into "infinite" chains, very similar to those observed in IP6-soaked crystals, where IP6 connects the concave sides of the N- and C-domains of adjacent protomers. In contrast, arrestin-3 only forms dimers, in which IP6 likely connects the C-domains of two arrestin-3 molecules. Thus, each of the three self-associating arrestins does it in its own way, forming three different types of

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oligomers. The physiological role of the oligomerization of arrestin-1 and both nonvisual arrestins might be quite different, and in each case it remains to be definitively elucidated.

Keywords Arrestin • Self-association • Structure • Crystal • EPR • Cytotoxicity

1 Visual Arrestin-1: The Discovery of Oligomerization

The beginning of arrestin history is rather convoluted: the first member of what we now call the arrestin family was originally discovered as an antigen against which patients with uveitis have antibodies (Wacker et al. 1977). Therefore, this protein was named S-antigen, and its gene is still called *Sag* in the HUGO database. The ability of this protein to oligomerize was described when it was identified, isolated, and characterized (Wacker et al. 1977). A soluble protein with an apparent molecular weight of ~48 kDa was later found to bind light-activated phosphorylated rhodopsin (P-Rh*) (Kuhn et al. 1984) and suppress its signaling (Wilden et al. 1986a). Later it was established that the 48-kDa protein and S-antigen are one and the same protein; it was named arrestin for its ability to "arrest" rhodopsin signaling. Despite active functional work with this protein, its oligomerization was largely ignored until two groups independently found that arrestin crystallizes as a tetramer under different conditions (Granzin et al. 1998; Hirsch et al. 1999) (Fig. 1). Its self-association was further analyzed by analytical centrifugation, which suggested that arrestin-1[1] forms dimers and tetramers in solution (Schubert et al. 1999). This was taken as an indication that the solution tetramer is likely similar to that in the crystal, and the data were interpreted accordingly (Schubert et al. 1999). Since it was clearly demonstrated earlier that at low nanomolar concentrations, where no self-association would be possible, arrestin-1 binds P-Rh* (Gurevich and Benovic 1992, 1993, 1995, 1997; Gurevich et al. 1995), oligomers were hypothesized to be an inactive storage form (Schubert et al. 1999). Two subsequent studies of arrestin-1 oligomerization by small-angle X-ray scattering yielded surprisingly different self-association constants (Imamoto et al. 2003; Shilton et al. 2002). Since the wavelength of X-rays is comparable to the size of arrestin, the small-angle X-ray scattering data could provide information about the shape of the solution tetramer, which was concluded to be the same as that in the crystal. One of these studies (Imamoto et al. 2003) proposed that visual

[1] Different systems of arrestin names are used in the field and in this book. We use the systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called "*arrestin 3*" in the HUGO database).

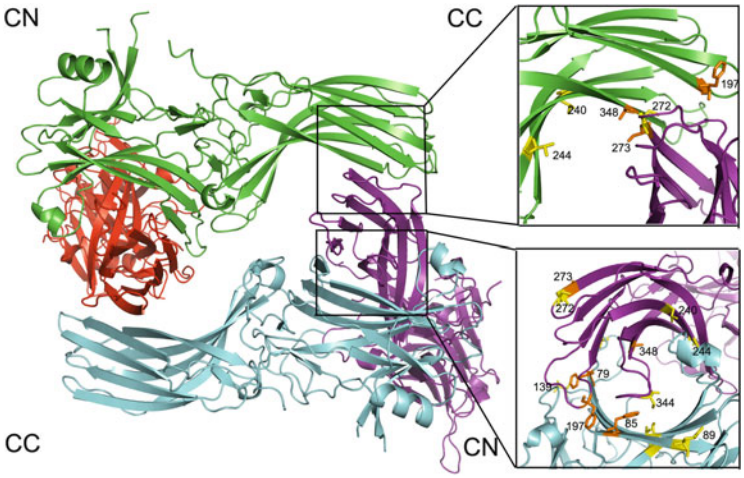

Fig. 1 The crystallographic tetramer of arrestin-1. In the structure [PDB ID: 1CF1 (Hirsch et al. 1999)] each protomer is shown in a *different color*. The crystallographic tetramer is a dimer of dimers, where individual dimers are held together via C-to-N-domain interfaces (CN), and the two dimers form a tetramer via C-to-C-domain interfaces (CC). The interfaces are enlarged on the *right*, with residues in positions probed by site-directed spin labeling EPR (Hanson et al. 2007c, 2008a) shown as *stick models*. Color coding: the residues in positions where the behavior of the spin label was consistent with predictions based on the crystal structure are shown in *orange*; those in positions where the behavior of the spin label was inconsistent with crystal structure are shown in *yellow*. Note that at least half of the positions fall into the latter category

arrestin-1 forms tetramers according to: $2M \leftrightarrows D$ (K_1), $2D \leftrightarrows T$ (K_2), where M, D, and T are monomer, dimer, and tetramer, respectively (MDT model). The oligomerization was found to be cooperative in the sense that the association constant $K_2 > K_1$. When the dimerization constant is much greater than the tetramerization constant, the concentration of dimers in the equilibrium mixture is small: it is dominated by tetramers.

2 Crystal and Solution Tetramers of Arrestin-1 Have Nothing in Common

Next, self-association of arrestin-1 in solution was analyzed by multi-angle laser light scattering (MALLS) (Hanson et al. 2007c). The advantages of this method include high resolution to within a few hundred Daltons, wide molecular mass range, relatively small sample size, and high sample throughput. Importantly, because the wavelength of light is large compared to the dimensions of arrestin-1 monomer or any oligomer, no assumptions regarding the shape of solution tetramer are necessary for data interpretation (Mogridge 2004). The results confirmed the earlier proposed MDT model (Imamoto et al. 2003) of monomer–dimer–tetramer

equilibrium and the cooperativity of self-association, although it yielded different constants for the same bovine arrestin-1: $K_1 = 2.7 \pm 0.1 \times 10^4$, $K_2 = 1.3 \pm 0.1 \times 10^5$, which translates into $K_{D,dim} = 1/K_1 = 37$ μM and $K_{D,tet} = 1/K_2 = 7.5$ μM (Hanson et al. 2007c). Interestingly, mutations that were predicted to disrupt self-association based on the crystal tetramer did not affect oligomerization, whereas many others that would not be expected to affect protomer interactions in the crystal had profound effects (Hanson et al. 2007c).

Continuous wave (CW) electron paramagnetic resonance (EPR) spectroscopy can be used to monitor the mobility of a spin label on the surface of a protein (Hanson et al. 2006b). If a particular element happens to be on the protomer–-protomer interaction interface, its mobility would decrease upon oligomer formation. A spin-label side chain (R1) introduced at many positions where significant immobilization was expected based on the crystal structure showed little to no change in mobility, whereas spin labels in several positions that are not on the crystal interfaces were immobilized upon tetramer formation (Hanson et al. 2007c). Collectively, the light scattering and EPR data showed that residues 79, 85, 173, 197, 244, and 348 are involved in inter-subunit interactions in the solution tetramer. While this result would be expected for 79, 85, 197, and 348 based on the crystal tetramer, the strong immobilization of 173R1 and the strong perturbation of self-association due to 244R1 were not predicted by the crystal tetramer (Fig. 1). Neither the native Leu173 and Val244 nor the R1 side chain modeled at these positions in the crystal tetramer makes contacts with neighboring subunits (Hirsch et al. 1999).

Relatively small perturbations and lack of immobilization of R1 at sites 60, 272, and 344, which are deeply buried at the CN, CC, and CN interfaces, respectively, were also inconsistent with the crystal tetramer, where residue 344 is buried to the extent that the R1 side chain cannot be modeled without major rearrangement of the structure (Fig. 1). Importantly, the 344R1 does not perturb the formation of oligomers (Hanson et al. 2007c). The weak perturbation of self-association by 89R1 and lack of spectral change of 89R1, located directly at the NN interface in the crystal (Fig. 1), do not support its existence. These results clearly indicate that the tetramer in solution is quite different from that observed in the crystal.

Double electron–electron resonance (DEER), a pulse EPR technique (Jeschke 2002), is a powerful method for measuring distances between paramagnetic centers in the range of ~19–60 Å (Pannier et al. 2000), complementing CW EPR methods that determine distances between 10 and 20 Å (Altenbach et al. 2001; Hanson et al. 2006b). DEER was used to measure distances between unique spin labels on each protomer within the solution tetramer, which were placed at eight non-perturbing or mildly perturbing sites in the tetramer (74, 108, 139, 173, 240, 272, 273, and 344). Only in one case (273R1) were the experimentally determined inter-spin distances close to the predictions based on the crystal structure, whereas the data for the other sites were clearly incompatible with the crystal tetramer (Hanson et al. 2007c). Thus, several lines of evidence independently suggested that the shape of the solution tetramer must be different.

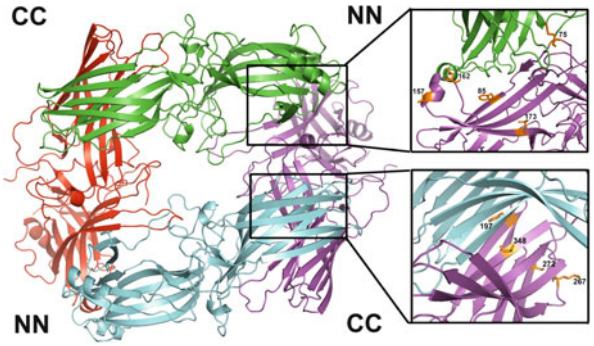

Fig. 2 Solution tetramer of arrestin-1. Studies using site-directed spin labeling EPR, long-range inter-subunit distance measurements by DEER spectroscopy, site-directed mutagenesis, Rosetta modeling, and inter-subunit disulfide bridge formation (Hanson et al. 2007c, 2008a) lead to the conclusion that the solution tetramer of arrestin-1 is a symmetrical closed diamond, where adjacent protomers interact via two types of interfaces: C-to-C domain (CC) and N-to-N domain (NN). Enlarged interfaces are shown on the *right*, with residues in positions experimentally tested by various methods shown as *stick models* (see text for details)

These unexpected findings made it necessary to elucidate the structure of the physiologically relevant solution tetramer, which holds clues to the functional role of arrestin-1 self-association. Since crystallography was misleading in this regard, the shape of the solution tetramer was deduced using inter-spin distances in the oligomer and the positions where the spin label was immobilized upon self-association (Hanson et al. 2007c, 2008a). These data were used as inputs for Rosetta modeling (Gray et al. 2003a, b; Schueler-Furman et al. 2005; Wang et al. 2005). Several iterations yielded a model for a tetramer consistent with all experimental data (Hanson et al. 2008a), which turned out to be symmetrical diamond shaped, with two nearly identical CC and NN interfaces, where all the interaction interfaces on each protomer are engaged by sister subunits (Fig. 2). Since modeling per se does not yield unambiguous information, this model was subjected to rigorous post hoc testing.

First, the R1 side chain was introduced either directly at the putative interface (position 75) or outside it (positions 376 and 381). CW EPR showed immobilization of the label at 75, with no evidence of immobilization at 376 or 381, consistent with the model (Hanson et al. 2008a). The residues Phe197 and Ala348 in the CC interface and Thr157 and Asp162 in the NN interface in the model are very close to their counterparts in the adjacent monomer (Fig. 2). In the crystal tetramer, all of these residues are far from their counterparts in other protomers (>20 Å). In contrast, residue Leu173 in the NN interface and Ser272 in the CC interface are far from their counterparts in the model. To test these predictions, single cysteine mutants were created and their ability to form inter-subunit disulfide bonds in solution was determined. In the presence of DTT, each arrestin ran as a single band on SDS-PAGE at a molecular weight (MW) corresponding to the arrestin monomer. However, in the absence of DTT, the Thr157Cys, Asp162Cys, Phe197Cys, and Ala348Cys mutants

showed a second band corresponding to the expected mobility of the arrestin dimer (Hanson et al. 2008a). This suggests that residues 157, 162, 197, and 348 are close enough to their counterparts in the arrestin oligomer to self-cross-link in solution. As predicted, the absence of DTT did not induce cross-linking of Leu173Cys and Ser272Cys. These data strongly support the orientation of the NN and CC interfaces in the model, since disulfide cross-linking only occurs at very short (~5 Å) Cβ–Cβ distances between the two residues.

Finally, the model was tested via targeted disruption of arrestin-1 self-association by mutations directly affecting predicted inter-subunit interfaces. Since the introduction of a spin label per se constitutes a mutation, first the effects of cysteine substitution followed by spin labeling at positions 85 in the predicted NN interface, as well as at positions 197 and 267 in the predicted CC interface, were evaluated. The labeling at all three positions reduced arrestin-1 self-association, confirming that these residues are in the inter-protomer interfaces. Importantly the effects of spin labeling at 197 and 267 were not additive, so that simultaneous labeling of both produced the same effect as the more detrimental to self-association 197R1 (Hanson et al. 2008a). This is consistent with both side chains being at the same interface. In contrast, the combination of 85R1 and 197R1 was much more disruptive than the labeling of either of these two sites alone, consistent with their localization in two different interfaces (Hanson et al. 2008a). Interestingly, the resulting 85R1/197R1 protein virtually lost the ability to self-associate. In the native structure both positions are occupied by phenylalanines. The replacement of Phe85 or Phe197 with alanine reduces self-association, whereas simultaneous substitution of both yields arrestin-1 that is essentially unable to oligomerize (Hanson et al. 2008a). Thus, three independent lines of evidence strongly support the model of solution tetramer (Fig. 2). Most importantly, these studies lead to the generation of a constitutively monomeric form of arrestin-1, which is necessary to elucidate the biological role of arrestin-1 self-association.

The proposed structure of the solution tetramer explains several observations that were inconsistent with the crystal tetramer. First, it explains the observed cooperativity (Hanson et al. 2007c; Imamoto et al. 2003): the interaction between two dimers engages two interfaces, whereas dimer formation involves only one. In contrast, in the crystal tetramer interfaces of comparable size mediate both dimerization and the interaction between two dimers in the tetramer (Granzin et al. 1998; Hirsch et al. 1999). Second, the circular "closed" configuration engages all self-association interfaces, explaining why arrestin-1 self-association stops at tetramer, so that larger oligomers are never formed. In contrast, in the crystal tetramer two protomers are left "dangling" with unused potential interaction interfaces (Granzin et al. 1998; Hirsch et al. 1999) that could mediate the binding of additional monomers. Finally, in the solution tetramer all arrestin-1 elements implicated in receptor binding, which were identified by numerous groups using a variety of methods (Dinculescu et al. 2002; Gimenez et al. 2012a; Gurevich and Benovic 1993; Gurevich et al. 1993; Hanson et al. 2006b; Hanson and Gurevich 2006; Kim et al. 2012; Ohguro et al. 1994; Pulvermuller et al. 2000; Vishnivetskiy et al. 2004, 2011; Zhuang et al. 2010, 2013), are either directly engaged or shielded by sister protomers,

which explains why only monomeric arrestin-1 can bind rhodopsin (Hanson et al. 2007c). Moreover, the proposed structure of the solution tetramer (Fig. 2) adequately explains the recent finding that manipulation of the receptor-binding surface of arrestin-1 to enhance its ability to interact with unphosphorylated rhodopsin significantly changes self-association parameters (Vishnivetskiy et al. 2013a).

3 The Mechanism of Arrestin-1 Self-Association Is Conserved in Mammalian Evolution

All these mechanistic studies were performed with bovine arrestin-1, which was purified first both from its native source (Wilden et al. 1986b) and upon overexpression in *Escherichia coli* (Gray-Keller et al. 1997). However, most of the physiological insights into rod function have been obtained in genetically modified mice (Arshavsky and Burns 2012; Makino et al. 2003), with the ultimate goal of translating the findings to human therapy (Song et al. 2009) (Chapter 7). The key biologically relevant facts about arrestin-1 were established in mice: (1) that it is the second (after rhodopsin) most abundant protein in rods (Hanson et al. 2007b; Song et al. 2011; Strissel et al. 2006) (see Chaps. 4 and 5); (2) that it undergoes light-dependent redistribution in rod photoreceptors (Hanson et al. 2007b; Nair et al. 2005) (Chaps. 4–6); and (3) that it is unexpectedly abundant in cones, where it represents ~98 % of total arrestin complement, whereas cone-specific arrestin-4 accounts for only ~2 % (Nikonov et al. 2008) (see Chap. 6). Thus, it was critically important to test whether mouse and human arrestin-1 self-associate and to determine the parameters of its oligomerization in these species.

Purified mouse arrestin-1 was found to form dimers and tetramers, similar to its bovine homolog (Kim et al. 2011). Interestingly, both dimerization ($K_{D,dim} = 57.5 \pm 0.6$ μM) and tetramerization ($K_{D,tet} = 63.1 \pm 2.6$ μM) dissociation constants of mouse protein were significantly higher than the corresponding values for bovine arrestin-1 [37.2 ± 0.2 μM and 7.4 ± 0.1 μM, respectively (Hanson et al. 2007c, 2008a)]. Moreover, whereas self-association of bovine arrestin-1 is cooperative ($K_{D,tet} < K_{D,dim}$) (Hanson et al. 2007c; Imamoto et al. 2003), both constants are roughly equal for mouse arrestin-1, eliminating cooperativity. The dramatic differences in self-association constants of arrestin-1 from these two mammalian species made it imperative to determine the properties of human arrestin-1. Purified human arrestin-1 was also found to self-associate and form dimers and tetramers. However, it demonstrated strikingly different constants compared to bovine and mouse proteins: remarkably low $K_{D,dim} = 2.95 \pm 0.02$ μM and relatively high $K_{D,tet} = 224 \pm 5$ μM (Kim et al. 2011). Importantly, if the overall concentration of arrestin-1 in the cell body of mammalian dark-adapted rod photoreceptors is similar to that measured in mouse (~2 mM (Song et al. 2011)), it greatly exceeds all measured dissociation constants. Therefore, despite these differences in self-association parameters the concentration of monomeric arrestin-1 in human, bovine, and mouse rods

would be in a fairly narrow range, 30–90 μM. As the majority of arrestin-1 would exist in the form of tetramer in all three species, the tetramer concentration in the rod would vary by no more than 30 %, and the most striking difference would be in the expected dimer concentrations, varying from ~60 μM in bovine to ~280 μM in human rod (Kim et al. 2011).

Nonetheless, measured $K_{D,dim}$ between human and mouse arrestin-1 differs ~20-fold, and $K_{D,tet}$ of bovine and human proteins is ~30-fold different. The magnitude of these differences raises the possibility that arrestin-1 in these three species could use distinct interaction interfaces. In this scenario phenomenological similarity of self-association could represent convergent evolution, rather than direct conservation of the molecular mechanism. The nature of the interaction interfaces in the solution tetramer of bovine arrestin-1 was strongly supported by the observation that the combination of two mutations predicted to disrupt NN (Phe85Ala) and CC (Phe197Ala) self-association interfaces makes the protein essentially a constitutive monomer, with $K_{D,dim} = 525$ μM and no detectable tetramerization (Hanson et al. 2008a). To test whether interaction interfaces are conserved in mouse protein, self-association of the double mutant carrying homologous substitutions Phe86Ala + Phe198Ala was tested. This mutation yielded the same phenotype as in bovine protein: mouse arrestin-1-Phe86Ala + Phe198Ala demonstrated dramatically impaired self-association, with $K_{D,dim} = 537$ μM and no tetramer formation (Kim et al. 2011). The finding that homologous mutations in bovine and mouse arrestin-1 similarly disrupt their self-association strongly suggests that both proteins use the same interfaces for oligomerization. Thus, strikingly different self-association constants reflect the difference in the energy of interactions between the subunits, whereas the organization of the solution tetramer is likely the same in all mammals. Importantly, the elimination of these two phenylalanines does not appreciably affect arrestin-1 binding to its two best-characterized partners, P-Rh* and microtubules (Kim et al. 2011). This finding suggests that these residues are strictly conserved in all mammalian arrestin-1 proteins (Gurevich and Gurevich 2006) because they facilitate self-association, indicating that robust arrestin-1 oligomerization is a biologically important aspect of its function in photoreceptor cells (Gurevich et al. 2011).

4 Possible Biological Role of Arrestin-1 Self-Association

Unambiguous demonstration that only monomeric arrestin-1 is capable of binding rhodopsin (Hanson et al. 2007c) confirmed the earlier hypothesis that dimers and tetramers are storage forms (Schubert et al. 1999). Although rod photoreceptors express many signaling proteins at levels several orders of magnitude higher than "normal" cells (Pugh and Lamb 2000), and arrestin-1 is the second most abundant protein in the rod (Hanson et al. 2007b; Song et al. 2011; Strissel et al. 2006), no other signaling protein in photoreceptors has an inactive storage form. Thus, arrestin-1 propensity to form inactive oligomers calls for an explanation.

The first glimpse into a possible role of this phenomenon emerged from unexpected quarters. In an attempt to compensate for defects in rhodopsin phosphorylation, two transgenic lines were created expressing the enhanced phosphorylation-independent arrestin-1-3A mutant (Gurevich 1998) at ~50 and ~240 % of normal WT level (Song et al. 2009). It turned out that the lower expressor line actually showed the expected compensation, whereas rod photoreceptors in the other degenerated even faster than in arrestin-1 knockout mice (Song et al. 2009, 2013). To achieve light sensitivity at the physical limit of single photons (Baylor et al. 1979), rods express very high levels of all signaling proteins (Pugh and Lamb 2000), maintaining a fairly precarious balance. As a result, overexpression of a perfectly normal WT protein can often lead to photoreceptor death, as has been shown for rhodopsin (Tan et al. 2001). However, it was found that the expression of WT arrestin-1 at essentially the same level, ~220 % of WT, is harmless (Song et al. 2011), indicating that it is the mutant nature of arrestin-1-3A that makes it toxic for rods. The analysis of the 3A mutant by MALLS showed that while this enhanced mouse arrestin-1 binds Rh* much better than WT, its self-association is partially compromised: $K_{D,dim}$ increased from 57.5 ± 0.6 μM of WT protein (Kim et al. 2011) to 135 ± 2 μM, with a simultaneous increase of $K_{D,tet}$ from 63.1 ± 2.6 μM to 380 ± 79 μM (Song et al. 2013). Calculations based on these constants, relative volumes of rod compartments (Peet et al. 2004), and arrestin-1 distribution in dark-adapted rod (Hanson et al. 2007b; Nair et al. 2005; Song et al. 2011; Strissel et al. 2006) indicate that the concentration of arrestin-1 monomer in the cell body of WT mouse rod is ~95 μM (out of ~2,000 μM of total arrestin-1). Due to robust self-association, a 2.2-fold increase of WT arrestin-1 to ~4,400 μM results in only a modest increase in free monomer, to ~104 μM. In contrast, the expression of arrestin-1-3A at 240 % of WT level would yield ~270 μM of monomer, almost three times more than in WT rods (Song et al. 2013). Importantly, the expression of the same mutant at ~50 % of WT level yields only ~115 μM monomer, which is not dramatically different from WT overexpressors, consistent with the relatively good health of photoreceptors in these animals (Song et al. 2009, 2013), at least until they reach the age of 32 weeks (Song et al. 2013). WT arrestin-1 was shown to effectively recruit mutants with partially compromised oligomerization into tetramers (Hanson et al. 2007c). Thus, if too high monomer concentration adversely affects rods, co-expression of WT arrestin-1 with the mutants would be expected to protect them. Indeed, it was shown that WT arrestin-1 expressed in rods with high levels of arrestin-1-3A affords partial protection against the mutant, slowing down photoreceptor death (Song et al. 2013). Interestingly, arrestin-1 was shown to interact with *N*-ethylmaleimide-sensitive factor (NSF) (Huang et al. 2010), a protein involved in exocytosis of neurotransmitter in the synapses. Indeed, synaptic terminals of rods expressing high levels of 3A mutant showed early damage, and their protection by WT arrestin-1 was very robust (Song et al. 2013). Collectively, the existing evidence is consistent with the idea that a relatively low level of monomeric arrestin-1 is optimal for photoreceptor health, whereas an excess of monomer induces cell death (see Chap. 16).

This hypothesis explains why arrestin-1 developed the ability to self-associate: rods need sufficient amounts of arrestin-1 to quench virtually all rhodopsin (Chap. 4 and 5), yet can tolerate only fairly low levels of monomer (Song et al. 2013). Thus, to solve this problem rods store the bulk of arrestin-1 in the form of "safe" oligomers. Cytotoxicity of the monomer can also explain the relatively low expression of arrestin-4 (Chan et al. 2007), which is outnumbered by arrestin-1 in cones by ~50:1 (Nikonov et al. 2008). Arrestin-4, a cone-specific subtype, appeared early in vertebrate evolution (Gurevich and Gurevich 2006). In contrast to other subtypes that form tight relatively long-lived complexes with their cognate GPCRs (Bayburt et al. 2011; Gurevich et al. 1995, 1997), arrestin-4 forms only low-affinity fairly transient complexes with cone opsins (Sutton et al. 2005). Functionally, this is perfectly suited for cones operating at high levels of illumination, which makes recycling and immediate reuse of cone opsins a necessity. Like rods, cones need enough arrestin to stop the signaling by all expressed photopigment. However, arrestin-4 is the only subtype that is self-association deficient, a natural constitutive monomer (Hanson et al. 2008b). If the monomer is toxic, cones simply cannot afford to express sufficient amounts of arrestin-4 and therefore keep the majority of their arrestin complement in the form of safely self-associating arrestin-1.

Thus, it appears that self-association of arrestin-1 is a cytoprotective mechanism, reducing the concentration of toxic monomer in photoreceptor cells. While it remains to be elucidated whether monomer toxicity arises from excessive binding to NSF (Huang et al. 2010), inappropriate engagement of clathrin adaptor AP2 (Moaven et al. 2013), or some other partner, it appears that arrestin-1 oligomerization prevents harmful interactions (Song et al. 2013).

5 Oligomerization of Nonvisual Arrestins: Mechanism and Consequences

Whereas arrestin-1 is expressed at very high levels in rods (Hanson et al. 2007b; Strissel et al. 2006) and cones (Nikonov et al. 2008), with concentrations reaching ~2 mM in the body of dark-adapted photoreceptors (Song et al. 2011), intracellular concentrations of nonvisual arrestins are much lower. Even in mature neurons, which express both at higher levels than most cells, the concentrations of arrestin-3 and -2 reach only ~30 and 200 nM, respectively (Gurevich et al. 2002, 2004). However, arrestins are fairly evenly distributed only in the non-stimulated cell (Song et al. 2006). Both arrestin-2 and -3 are recruited to active phosphorylated GPCRs in all cell types and were shown to become concentrated in the vicinity of the plasma membrane and endosomes upon GPCR activation (Barak et al. 1997). By virtue of their binding to polymerized tubulin (Hanson et al. 2006a), nonvisual arrestins also appear to be concentrated in the vicinity of microtubules (Hanson et al. 2007a). Thus, local concentration in particular cell compartments under certain circumstances can greatly exceed estimated averages. Indeed, arrestin-2

and -3 expressed at near-physiological levels were reported to form homo- and hetero-oligomers in cells (Milano et al. 2006; Storez et al. 2005). Hetero-oligomerization of arrestin-3, which has a functional nuclear export signal in it C-terminus (Scott et al. 2002; Song et al. 2006; Wang et al. 2003), with arrestin-2 that does not, appears to help the removal of arrestin-2 from the nucleus (Storez et al. 2005).

Inositol-hexakisphosphate (IP6, a.k.a. phytic acid), an abundant metabolite present in many cells in concentrations of 15–100 μM (Shears 2001), was shown to greatly enhance self-association of both nonvisual arrestins (Milano et al. 2006). Even though full-length arrestin-2 crystallizes as a monomer (Milano et al. 2002), solving the structure of crystals soaked with IP6 revealed that IP6 bridges neighboring molecules in a head-to-tail configuration via interactions with two sites, one in the N-domain and the other in the C-domain of arrestin-2 (Milano et al. 2006). Direct binding studies combined with extensive mutagenesis showed that the C-domain site has a much higher affinity (K_D ~ 40 nM) than the N-domain site (K_D ~ 1 μM) for IP6, but both are well within the range of physiological IP6 concentrations in the cell (Milano et al. 2006).

Elimination of positively charged residues critical for IP6 binding increased the arrestin-2 presence in the nucleus, suggesting that oligomers are largely cytoplasmic (Milano et al. 2006). Both IP6 binding sites appear to be localized on the receptor-binding surface of arrestins identified by many groups using various methods (Dinculescu et al. 2002; Gimenez et al. 2012b; Gurevich and Benovic 1993, 1995; Gurevich et al. 1995; Hanson et al. 2006b; Hanson and Gurevich 2006; Kim et al. 2012; Ohguro et al. 1994; Pulvermuller et al. 2000; Vishnivetskiy et al. 2011; Zhuang et al. 2013), indicating that simultaneous binding of receptor and IP6 is impossible. This finding suggested that, as in the case of arrestin-1, oligomers represent an inactive storage form, whereas monomeric arrestins are recruited to GPCRs, as well as translocated to the nucleus (Milano et al. 2006). Interestingly, while IP6 greatly increases self-association of nonvisual subtypes (Hanson et al. 2008b; Milano et al. 2006), it significantly inhibits the oligomerization of arrestin-1 (Hanson et al. 2008b), indicating that the interfaces involved and overall shape of the oligomers formed by visual and nonvisual arrestins are different.

Experiments with purified proteins and cells expressing IP6 binding-deficient mutants of both arrestin-2 and -3 also suggested that they form oligomers larger than dimer (Milano et al. 2006). However, while arrestin-1 was shown to stop at tetramer (Hanson et al. 2007c), in which all interaction interfaces are engaged by sister subunits (Hanson et al. 2008a), it remained unclear whether arrestin-2 and -3 also stop at a particular size of oligomer, or can form “infinite” chains, as suggested by IP6-soaked arrestin-2 crystal structure (Milano et al. 2006). This issue was addressed using pure arrestins in the presence of IP6 by MALLS (Chen et al. 2013). In the absence of IP6, arrestin-2 and -3 have a low tendency to self-associate with a K_D around 100 μM (Fig. 3). IP6 promotes their self-association, and the K_Ds decrease to 5.5 and 7.8 μM for arrestin-2 and -3, respectively (Chen et al. 2013).

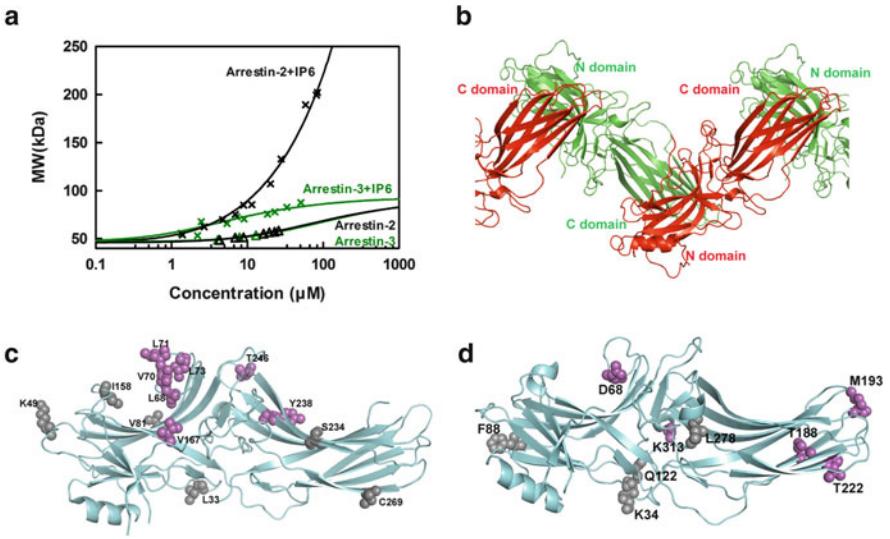

Fig. 3 The two nonvisual arrestins form distinct oligomers. (**a**) The average molecular weight of arrestin-2 and arrestin-3 in the presence (*crosses*) and absence (*triangles*) of 100 μM IP6 as a function of total arrestin concentration was measured by MALLS. Arrestin-2 data were fit by a linear polymerization model (*black line*), while arrestin-3 data were fit by a monomer–dimer model (*green line*). Neither nonvisual arrestin showed a propensity to self-associate at physiologically relevant concentrations in the absence of IP6. (**b**) The crystal structure of arrestin-2 in complex with IP6 [PDB ID: 1ZSH (Milano et al. 2006)] shows that arrestin-2 forms "infinite" chains through C-to-N-domain interactions mediated by IP6. (**c**) The positions of spin-labeled sites are shown as *spheres* on the crystal structure of arrestin-2 [PDB ID: 1ZSH (Milano et al. 2006)]. The sites with inter-subunit distances shorter than 50 Å (Leu68, Val70, Leu71, Leu73, Val167, Tyr238, and Thr246), as measured by DEER spectroscopy in the presence of IP6, are colored *magenta*, and the ones with inter-subunit distance longer than 50 Å (Leu33, Lys49, Val81, Ile158, Ser234, and Cys269) are colored *gray* [data from Chen et al. (2013)]. (**d**) The positions of spin-labeled sites are shown as *spheres* on the crystal structure of arrestin-3 [PDBID: 3P2D (Zhan et al. 2011)]. The sites with inter-subunit distance shorter than 50 Å (Asp68, Thr188, Met193, Thr222, and Lys313), as measured by DEER spectroscopy in the presence of IP6, are colored *magenta*, while the ones with inter-subunit distance longer than 50 Å (Lys34, Phe88, Gln122, and Leu278) are colored *gray* [data from Chen et al. (2013)]

Despite high homology arrestin-2 and -3 form distinct oligomers in the presence of IP6: arrestin-3 forms dimers; in contrast, arrestin-2 forms long chains that go beyond tetramer. The average molecular weight of arrestin-2 keeps growing without obvious saturation. At the highest concentration tested (84 μM), the average molecular weight of arrestin-2 oligomers reached ~202 kDa, which exceeded the expected molecular weight for the arrestin-2 tetramer (184 kDa) (Fig. 3). Due to the formation of higher order oligomers MALLS data do not fit into the MDT model. Instead, a liner polymerization model ($M[n] + M \leftrightarrow M[n + 1]$) fit arrestin-2 oligomerization data very well (Chen et al. 2013). This suggested that arrestin-2 might form an infinite chain mediated by IP6, as in the arrestin-2 crystals soaked with

IP6 (Milano et al. 2006). However, the crystal structure does not necessarily reflect that which exists in solution, since in that study the orientation of arrestin-2 molecules relative to each other was fixed by crystallization in the absence of IP6 (Milano et al. 2006). Therefore, DEER was used to probe the structure of the solution oligomer of arrestin-2 in the presence of IP6 (Chen et al. 2013). Thirteen sites on arrestin-2 were selected on both the N-domain (Leu33, Lys49, Leu68, Val70, Leu71, Leu73, Val81, Ile158, and Val167) and the C-domain (Ser234, Tyr238, Thr246, and Cys269). These sites were located on the receptor-binding concave side (Lys49, Ile158, Val81, Leu68, Val70, Leu71, Leu73, Val167, Thr246, Tyr238, and Ser234) and the convex side (Leu33 and Cys269) to obtain a comprehensive characterization of the solution oligomer of arrestin-2 in the presence of IP6. A nitroxide spin label (R1) at selected sites in arrestin-2 was introduced by chemical modification of these unique cysteines and the inter-subunit distances were measured using the DEER spectroscopy. The measured DEER distances (Chen et al. 2013) matched remarkably well with the expected nitroxide-to-nitroxide distances between adjoining protomers in the crystal structure (Milano et al. 2006). All but two sets of data matched to within 3 Å of the expected crystallographic distances. Importantly, the sites with closer distances (<50 Å) clustered in the central parts of the concave receptor-binding side, which suggested that IP6 mediates the interaction between the N- and C-domains of arrestin-2, so that only the central parts of the concave side come close together (Fig. 3). Collectively, these data clearly suggest that the arrangement of protomers in the arrestin-2 crystals soaked with IP6 closely resembles the structure of the solution oligomer of arrestin-2, further supporting the hypothesis that arrestin-2 forms "infinite" chains in the presence of IP6 in solution, similar to those observed in the crystal (Chen et al. 2013; Milano et al. 2006). In contrast, MALLS data showed that the average molecular weight of arrestin-3 oligomers in the presence of IP6 did not exceed that of a dimer (Chen et al. 2013) (Fig. 3). Since the saturation was not reached due to concentration limitations, a higher order oligomer could not be excluded. However, the fact that the data could not be fit to either an MDT model or a polymerization model, but fit well to monomer–dimer equilibrium model suggested that the formation of higher order oligomers of arrestin-3 in the presence of IP6 was not favored. DEER was used to probe the structure of arrestin-3 oligomers in solution in the presence of IP6. The distances measured with several arrestin-3 mutants in the presence of IP6 aligned moderately well with the expected distances based on the arrestin-2 IP6 crystallographic oligomer, but they were not as clearly matched as the arrestin-2 data (Chen et al. 2013). Interestingly, the sites in arrestin-3 with distances shorter than 50 Å are clustered not only in the central crest (Asp68, Lys313), but also in the C-domain, including the distal part of the C-domain (Thr188, Met193, Thr222), while the sites in the N-domain (Lys34, Phe88, and Gln122) had much longer distances, beyond the range of reliable measurement by DEER spectroscopy (Chen et al. 2013). These data suggested that IP6 might mediate the interaction between the C-domains of two arrestin-3 molecules, so that the sites on the C-domain are in close contact, whereas the sites

on the N-domain remain far apart. This model would explain why arrestin-3 stops at a dimer, since the interfaces mediating IP6-assisted interaction are no longer exposed upon the formation of the C-to-C-domain dimer. This is in contrast to arrestin-2, in which only the sites in the central crest on the concave side come close to each other in the presence of IP6. Though more data are needed to generate a high-resolution model for the arrestin-3 dimer (or larger oligomer, if it exists), it is very clear that in the presence of IP6 arrestin-2 and arrestin-3 form structurally distinct oligomers (Chen et al. 2013). Since nonvisual arrestins were reported to form mixed oligomers (Milano et al. 2006; Storez et al. 2005), it remains to be elucidated whether these resemble arrestin-2 chains or arrestin-3 C-to-C dimers or have a unique shape and size distinct from both.

6 Do Arrestin Oligomers Have Specific Functions?

It was shown that mutations disrupting self-association of arrestin-1 do not significantly affect its ability to bind its preferred form of rhodopsin, P-Rh*, or microtubules (Kim et al. 2011). However, the same mutations somewhat reduced the binding of an enhanced phosphorylation-independent mutant to Rh* (Vishnivetskiy et al. 2013a), in agreement with the finding that distinct arrestin-1 elements are involved in its interactions with different functional forms of rhodopsin (Zhuang et al. 2013). This difference might also reflect distinct stoichiometry of the arrestin-1–rhodopsin interactions in these cases. While arrestin-1 was shown to bind the P-Rh* monomer in nanodiscs (Bayburt et al. 2011; Kim et al. 2012; Singhal et al. 2013; Vishnivetskiy et al. 2013b) and bicelles (Zhuang et al. 2013), a possibility of an alternative mode of interaction was reported in native disc membranes with a high fraction of light-activated rhodopsin, where arrestin-1 appears to engage two rhodopsin molecules simultaneously (Sommer et al. 2011, 2012) (Chap. 5). Even though in these situations arrestin-1 binds only one rhodopsin molecule with high enough affinity to stabilize its active conformation (Sommer et al. 2011, 2012), the engagement of one or two rhodopsin molecules, one of which might be unphosphorylated, could be one of the mechanistic differences in arrestin-1 binding to P-Rh* and Rh*. In either case, it appears that only monomeric arrestin-1 can bind rhodopsin. Interestingly, while rhodopsin binding induces the dissociation of all arrestin-1 oligomers, indicating that only monomeric arrestin-1 can bind the receptor (Hanson et al. 2007c), the monomer (Hanson et al. 2006a, 2007a) and all oligomers appear to bind tubulin comparably, so that in the presence of a sufficient concentration of microtubules to bind all arrestin-1 the inter-subunit distances reporting the presence of oligomers do not appear to be affected (Hanson et al. 2007c).

In the case of nonvisual arrestins, we know even less about specific functions of the oligomeric forms. Oligomerization-deficient mutants were found to bind clathrin, clathrin adaptor AP2, and ERK1/2 normally (Milano et al. 2006), in agreement with the localization of binding sites for these partners (Coffa et al. 2011;

Goodman et al. 1996; Laporte et al. 1999) away from residues that mediate IP6 binding (Milano et al. 2006). However, an arrestin-3 mutant that did not bind IP6 was found to lack tight association with another partner, ubiquitin ligase Mdm2, and in contrast to WT arrestin-3, these presumably monomeric mutants did not suppress Mdm2-dependent degradation of p53 (Boularan et al. 2007). While it was proposed that arrestin-3 oligomers provide more interaction sites for putative dimers of Mdm2 (Boularan et al. 2007), another plausible explanation is that since Mdm2 preferentially binds arrestins in the basal conformation (Ahmed et al. 2011; Song et al. 2006, 2007), oligomerization might simply stabilize this conformational state of nonvisual arrestins, which are inherently more flexible than arrestin-1 (Han et al. 2001; Hirsch et al. 1999; Zhan et al. 2011). One study suggested that monomeric nonvisual arrestins are more likely to enter the nucleus (Milano et al. 2006), whereas another found comparable levels of arrestin oligomers in the nucleus and cytoplasm (Boularan et al. 2007), although in the latter case it remained unclear whether arrestin oligomers can enter the nucleus, or arrestins self-associate after entering it as monomers and/or dimers.

It is entirely possible that nonvisual arrestins self-associate for the same reason as arrestin-1 to prevent the buildup of a cytotoxic monomeric form (Song et al. 2013), but this idea needs to be tested experimentally. It is clear that more experimentation is necessary before we will be able to unambiguously determine specific functions of nonvisual arrestin oligomers and sort out cellular processes affected by their impaired self-association.

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Arrestin-Dependent Activation of ERK and Src Family Kinases

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Abstract The four members of the mammalian arrestin family, two visual and two nonvisual, share the property of stimulus-dependent docking to G protein-coupled receptors. This conformational selectivity permits them to function in receptor desensitization, as arrestin binding sterically inhibits G protein coupling. The two nonvisual arrestins further act as adapter proteins, linking receptors to the clathrin-dependent endocytic machinery and regulating receptor sequestration, intracellular trafficking, recycling, and degradation. Arrestins also function as ligand-regulated scaffolds, recruiting catalytically active proteins into receptor-based multiprotein "signalsome" complexes. Arrestin binding thus marks the transition from a transient G protein-coupled state on the plasma membrane to a persistent arrestin-coupled state that continues to signal as the receptor internalizes. Two of the earliest discovered and most studied arrestin-dependent signaling pathways involve regulation of Src family nonreceptor tyrosine kinases and the ERK1/2 mitogen-activated kinase cascade. In each case, arrestin scaffolding imposes constraints on kinase activity that dictate signal duration and substrate specificity. Evidence suggests that arrestin-bound ERK1/2 and Src not only play regulatory roles in receptor desensitization and trafficking but also mediate longer term effects on cell growth, migration, proliferation, and survival.

Keywords Arrestin • Extracellular signal-regulated kinase • G protein-coupled receptor • Signal transduction • Src family nonreceptor tyrosine kinase

Abbreviations

BRET	Bioluminescence resonance energy transfer
EGF	Epidermal growth factor
ERK1/2	Extracellular signal-regulated kinases 1 and 2
GPCR	G protein-coupled receptor
GRK	GPCR kinase
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MEF	Murine embryo fibroblast
PAR	Protease-activated receptor
PK	Protein kinase
PLC	Phospholipase C
PTH	Parathyroid hormone
SH	Src homology

1 Arrestins as Ligand-Regulated GPCR Scaffolds

Heptahelical G protein-coupled receptors (GPCRs) function as ligand-activated guanine nucleotide exchange factors for heterotrimeric G proteins. Agonist binding stabilizes the receptor in an "active" conformation wherein it catalyzes the exchange of GTP for GDP on heterotrimeric G protein Gα subunits, leading to conformational rearrangements between the GTP-bound Gα subunit and the Gβγ subunit heterodimer. Once dissociated, Gα-GTP and Gβγ subunits regulate the activity of enzymatic effectors, such as adenylyl cyclases, phospholipase C (PLC) isoforms, and ion channels, generating small molecule second messengers that control the activity of enzymes involved in intermediary metabolism.

Predictably, G protein-mediated signaling is subject to extensive negative regulation. Second messengers are rapidly inactivated by cyclic nucleotide phosphodiesterases, phosphatidylinositol phosphatases, diacylglycerol kinases, and the reuptake and extrusion of cytosolic calcium. G protein activity is limited by the intrinsic GTPase activity of Gα subunits and the extrinsic action of regulators of G protein signaling (RGS) proteins, which function as GTPase-activating proteins to return G proteins to their inactive heterotrimeric state (Ross and Wilkie 2000). Receptor-G protein coupling is controlled by phosphorylation. Second messenger-dependent protein kinases like protein kinase (PK)A and PKC mediate heterologous desensitization, so named because it does not require ligand occupancy. Phosphorylation of intracellular receptor domains by these kinases is sufficient to impair receptor-G protein coupling without the involvement of accessory proteins (Freedman and Lefkowitz 1996). In contrast, homologous desensitization is both sensitive to receptor conformation and dependent on the binding of arrestins. G protein-coupled receptor kinases (GRK)1–7 phosphorylate agonist-occupied receptors on serine or threonine residues in the receptor C terminus or the third intracellular loop (Stoffel et al. 1997). GRK-phosphorylated receptors recruit arrestins, which translocate from the cytosol to the plasma membrane to physically interdict receptor-G protein coupling. The two nonvisual arrestins, arrestin2 (β-arrestin1) and arrestin3 (β-arrestin2), further diminish signaling by acting as adapter proteins that link receptors to the clathrin-dependent endocytic machinery (Ferguson 2001). Arrestin-dependent sequestration limits signal duration; removes receptors from the cell surface, rendering it less responsive to subsequent stimuli; and ultimately determines whether receptors "resensitize" and recycle to the cell surface or undergo degradation (*see* chapters "Arrestin Interactions with G Protein-Coupled Receptors," "β-Arrestins and G Protein-Coupled Receptor Trafficking," and "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications").

Although the ability of arrestins to act as ligand-regulated adapter proteins was first appreciated in the context of GPCR desensitization and sequestration, it was the subsequent discovery that the arrestins bind catalytically active proteins and redistribute them from the cytosol to the plasma membrane as they dock with GRK-phosphorylated receptors that led to a paradigm shift in GPCR biology

(Luttrell and Lefkowitz 2002; Luttrell and Gesty-Palmer 2010). The capacity to function as ligand-regulated scaffolds enables arrestins to nucleate the formation of multiprotein "signalsomes" linking GPCRs to novel non-G protein effectors, among them protein and lipid kinases, phosphatases, phosphodiesterases, ubiquitin ligases, and regulators of small G proteins. Arrestin binding, then, does not mark the end of GPCR signaling, but the transition from one receptor signaling state to another. The concept of "pluridimensional efficacy" (Galandrin and Bouvier 2006) that arose from the discovery of arrestin-dependent signaling recognizes that GPCRs signal via both G protein and non-G protein effectors that in sum comprise the signaling repertoire of these versatile receptors. The further demonstration that G protein- and arrestin-mediated signals are not only mechanistically independent, but also pharmacologically dissociable (Wei et al. 2003), raises the prospect of "biased" therapeutics that tailor GPCR signaling to elicit desired responses while simultaneously antagonizing maladaptive ones (Maudsley et al. 2005; Kenakin and Miller 2010; Luttrell and Kenakin 2011; *see* chapter "Quantifying Biased β-Arrestin Signaling").

Among the first discovered arrestin-dependent signals were the regulation of Src family nonreceptor tyrosine kinases and the extracellular signal-regulated kinases 1 and 2 (ERK1/2). In each case, arrestin-dependent recruitment enables GPCRs to regulate a pool of kinase activity with distinct spatial and temporal characteristics that target them to perform specific functions. Both arrestin-regulated ERK1/2 and Src contribute to the control of GPCR endocytosis and trafficking by phosphorylating key regulatory proteins, such as GRKs and dynamin. Moreover, both kinases link GPCRs to longer term processes related to cell growth, proliferation, survival, and migration. In this chapter, we examine the mechanism of GPCR regulation of these two important arrestin binding partners and discuss their physiologic roles in GPCR regulation and cell biology.

2 Arrestin-Dependent Activation of ERK1/2

The duality of arrestin function can be illustrated by a simple experiment. Overexpression of either arrestin2 or 3 in COS-7 cells expressing the angiotensin AT_{1A} receptor blunts angiotensin II-stimulated inositol phosphate production via the $G_{q/11}$-PLCβ effector pathway, due to accelerated uncoupling of the heterotrimeric G protein from its receptor. But the same conditions produce a paradoxical increase in AT_{1A} receptor-mediated ERK1/2 activation, indicating that arrestins enhance coupling to some downstream effectors while impairing others (Tohgo et al. 2002). The physical basis of this paradox lies in the ability of arrestins to act as GPCR effectors and mediate G protein-independent ERK1/2 activation.

2.1 Mitogen-Activated Protein Kinases

The mitogen-activated protein kinases (MAPK) are a family of evolutionarily conserved serine/threonine kinases involved in the transduction of externally derived signals regulating cell growth, division, differentiation, and apoptosis. Mammalian cells express three major classes of MAPK: the extracellular signal-regulated kinases, ERK1/2; the c-Jun N-terminal kinase/Stress-activated protein kinases (JNK/SAPK); and the p38/HOG1 MAPKs. The ERK1/2 pathway is pleiotropic, but critically involved in growth factor-promoted G0–G1 cell cycle transition. In contrast, the JNK/SAPK and p38/HOG1 MAPKs are principally involved in growth arrest and apoptosis in response to environmental and hormonal stresses (Kryiakis and Avruch 1996; Pearson et al. 2001).

MAPK activity in cells is organized into a series of parallel kinase cascades, each composed of three kinases that successively phosphorylate and activate the downstream component. In the ERK1/2 cascade, the proximal kinases, cRaf-1 and B-Raf (MAPK kinase kinases), phosphorylate and activate MEK1 and MEK2 (MAPK kinases). MEK 1 and 2 are dual function threonine/tyrosine kinases that, in turn, carry out the phosphorylation and activation of ERK1/2. Once activated, ERK1/2 phosphorylates a variety of membrane, cytoplasmic, nuclear, and cytoskeletal substrates. Active ERK1/2 also translocates to the nucleus, where it phosphorylates and activates nuclear transcription factors involved in DNA synthesis and cell cycle progression (Pearson et al. 2001).

In many cases, MAPK activity is regulated by binding of the component kinases to a scaffolding protein (Burack and Shaw 2000). These scaffolds serve at least three functions in cells: to increase the efficiency of signaling between successive kinases in the cascade, to ensure signaling fidelity by dampening cross talk between parallel MAPK cascades, and to target MAPKs to specific subcellular locations. The prototypic MAPK scaffold is the *Saccharomyces cerevisiae* protein, Ste5p (Choi et al. 1994). In the yeast pheromone mating pathway, Ste5p binds to Ste11p (MAPK kinase kinase), to Ste7p (MAPK kinase), and to either Fus3p or Kss1p (MAPK). Binding of yeast mating factor to the pheromone receptor, a GPCR, triggers heterotrimeric G protein activation. Subsequent translocation of Ste5p to the plasma membrane in response to the release of Gβγ subunits leads to activation of the Fus3/Kss1 cascade. While no structural homologues of Ste5p exist mammalian cells, several mammalian proteins that can bind to two or more components of a MAPK module have been identified. For example, the JIP family of proteins acts as scaffolds for regulation of the JNK/SAPK pathway (Whitmarsh et al. 1998). Importantly, arrestins, despite the lack of structural homology with Ste5p, function in an analogous manner, acting as GPCR-regulated scaffolds for MAPK activation. In addition to regulating ERK1/2, arrestins have been implicated in the positive or negative regulation of the JNK/SAPK and p38/HOG1 cascades (*see* chapters "Arrestin-Dependent Activation of JNK Family Kinases" and "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences," respectively).

2.2 Binding and Activating the Raf–MEK–ERK Cascade

In contrast to the clathrin and AP-2 binding sites in the C terminus of arrestins 2 and 3 (*see* chapter "β-Arrestins and G Protein-Coupled Receptor Trafficking"), the binding sites for ERK1/2 cascade components on arrestins have not been precisely mapped. Indeed, most data suggest that c-Raf1, MEK1/2, and ERK1/2 make multiple contacts with the exposed cytosolic face of the receptor-bound arrestin, a feature that may underlie its ability to catalyze receptor-dependent ERK1/2 activation (Song et al. 2009). The initial observations, based on co-immunoprecipitation from cells transfected with pathway components, were that while all ERK1/2 pathway kinases interacted with arrestin3, binding of MEK1 and ERK2 was enhanced by co-expressed c-Raf1, suggesting a cooperative binding interaction. Moreover, activation of angiotensin AT_{1A} receptors increased c-Raf1 and ERK2 binding to arrestin3, and the association of all three proteins with the receptor, consistent with ligand-dependent complex assembly (Luttrell et al. 2001). Subsequent work demonstrated that c-Raf1, MEK1, and ERK2 each bound to the separately expressed N- and C domains of all four arrestin isoforms, suggesting that the binding sites for all three kinases are bipartite and that binding per se is not the key to arrestin-mediated pathway activation (Song et al. 2009).

Using conformationally biased arrestin mutants that mimic the free, receptor-bound, and microtubule-associated conformations of arrestins, it has been possible to show that ERK1/2 interacts with high affinity only with the receptor-bound and microtubule-associated conformations and exhibits virtually no binding to free cytosolic arrestin (Coffa et al. 2011a). Like ERK1/2, c-Raf1 prefers the receptor-bound conformation, although the difference is less dramatic, while MEK1 binds equivalently to both free arrestin (Meng et al. 2009; Coffa et al. 2011b) and all three mutationally constrained conformations (Coffa et al. 2011a). Perhaps significantly, cRaf1 and ERK1/2 binding to the microtubule-bound pool of arrestin may provide a mechanism for dampening basal ERK1/2 activity in the absence of receptor stimulation. In cells, overexpressed arrestins1, 2, and 3, but not arrestin4, recruit ERK1/2 to microtubules and quench its activity (Hanson et al. 2007), as does the arrestin3 mutant that mimics the microtubule-associated conformation (Coffa et al. 2011a). On the other hand, arrestin-dependent activation of ERK1/2 appears to be dependent upon binding to GPCRs (Coffa et al. 2011a, b), as originally proposed (Luttrell et al. 2001).

The situation appears quite different for the related Ask1–MKK4–JNK3 cascade. All four arrestin isoforms bind the component kinases equivalently, but only arrestin3 efficiently activates JNK3 (Miller et al. 2001; Seo et al. 2011). Although it was originally proposed that arrestin3-mediated JNK3 activation was receptor dependent (McDonald et al. 2000), subsequent work showed that wild-type arrestin3, a pre-activated phosphorylation-independent mutant, and a mutant with impaired receptor binding were all equally effective at both activating JNK3 and sequestering it in the cytosol away from its nuclear substrates (Scott et al. 2002; Song et al. 2006; Breitman et al. 2012). Such findings suggest that rather than

serving as ligand-regulated activators of JNK3, arrestins may behave as a "silent scaffolds," either by acting as endogenous inhibitors of JNK3 signaling or by targeting active JNK3 toward nonnuclear substrates (Breitman et al. 2012).

Agonist binding to GPCRs like the protease-activated receptor (PAR)2 and angiotensin AT_{1A} receptor induces the assembly of a stable "signalsome" complex containing the receptor, arrestin, c-Raf1, MEK1/2, and activated ERK1/2 (DeFea et al. 2000a; Luttrell et al. 2001; Tohgo et al. 2002, 2003). This arrestin-mediated signal does not require heterotrimeric G protein activation, as it can be generated by a mutant AT_{1A} receptor that lacks G protein-coupling efficacy and by a "biased" AT_{1A} receptor agonist, [Sar^1-Ile^4-Ile^8]-AngII, that promotes arrestin recruitment and receptor internalization without G protein activation (Wei et al. 2003). Indeed, the principal role of the receptor appears to be to provide a ligand-regulated arrestin docking site that elicits the conformational changes in the arrestin required for efficient ERK1/2 binding and complex assembly. Membrane translocation may also play a role. Expression of a G protein-uncoupled neurokinin NK1 receptor-arrestin2 chimera leads to constitutive activation of a pool of ERK1/2 that remains bound, along with c-Raf1 and MEK1/2, to the endosomal membrane-delimited receptor-arrestin chimera (Jafri et al. 2006). Since membrane targeting of c-Raf1 is sufficient to activate ERK1/2 (Stokoe et al. 1994), one possibility is that the arrestin functions simply to move cytosolic c-Raf1 to the membrane for activation. The finding that plasma membrane recruitment of arrestin3 independent of receptor binding is sufficient to activate ERK1/2, albeit inefficiently, is consistent with this model (Terrillon and Bouvier 2004). ERK1/2 bound to the signalsome complex is also protected from dephosphorylation by MAPK phosphatases, suggesting that a slower rate of inactivation also promotes sustained ERK1/2 activity (Jafri et al. 2006).

2.3 Temporal and Spatial Regulation of ERK Activity

As depicted schematically in Fig. 1, ERK1/2 is subject to extensive convergent regulation, including Ras-dependent signals originating from GPCRs and receptor tyrosine kinases, PKA- and PKC-mediated signals downstream of heterotrimeric G proteins, and signals transmitted via arrestin scaffolds (Pierce et al. 2001a; Luttrell 2003). While receptor and cell-type variability in the mechanisms of G protein-dependent ERK1/2 activation is more the rule than the exception, it is clear that most heterotrimeric G protein families also signal to ERK1/2. Stimulation of the $G_{q/11}$–PLCβ–PKC pathway can activate ERK1/2 through direct phosphorylation of c-Raf1 by PKCα (Kolch et al. 1993; Hawes et al. 1995). The consequences of stimulating of cAMP production by G_s-adenylyl cyclase are complex and determined primarily by which Raf isoform, c-Raf1 or B-Raf, is predominantly expressed. Unlike PKC, phosphorylation of cRaf-1 by PKA inhibits its activation (Wu et al. 1993). As a result, activation of G_s-coupled receptors in some cells inhibits, rather than activates, ERK1/2 (Lefkowitz et al. 2002). On the other hand,

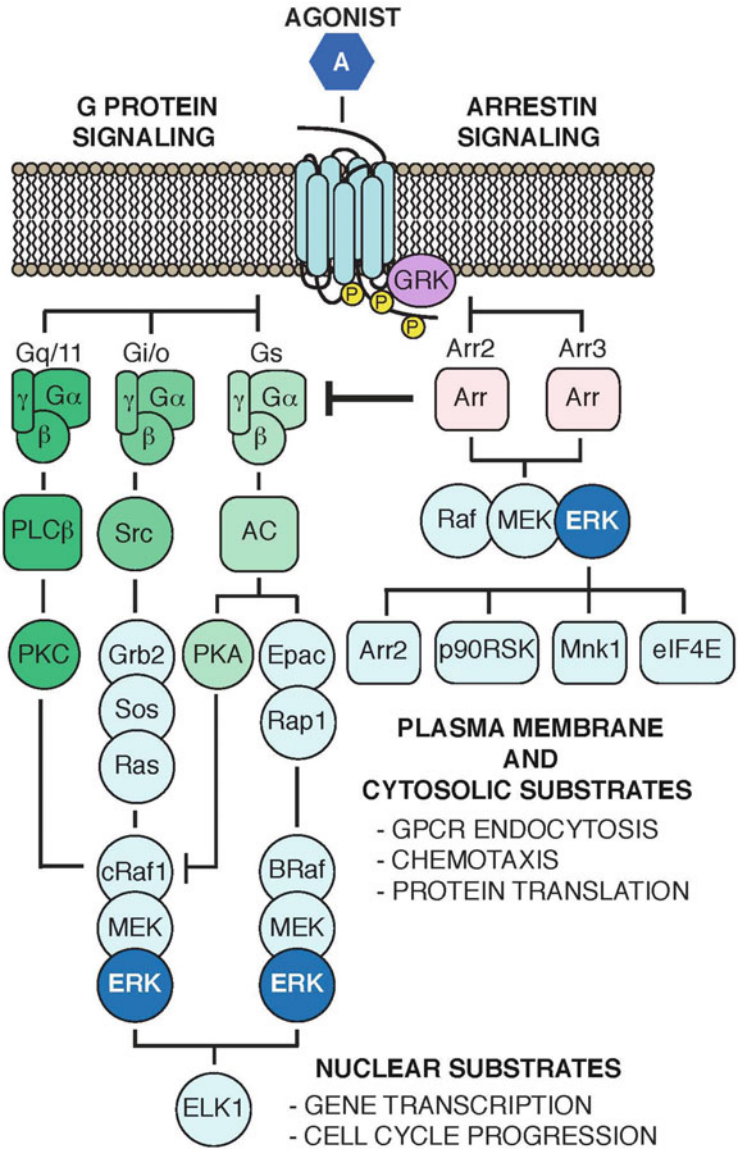

Fig. 1 Arrestins regulate a complex web of GPCR-ERK1/2 signaling. Upon agonist (A) binding, GPCRs engage both G protein- and arrestin-mediated pathways to control ERK1/2 activity. G protein-mediated signals from $G_{q/11}$, $G_{i/o}$, and G_s proteins converge on the two predominant Raf isoforms, cRaf1 and B-Raf, to activate the Raf-MEK-ERK kinase cascade. Stimulation of $G_{q/11}$-PLCβ-PKC can produce direct PKC-mediated activation of cRaf-1. $G_{\beta\gamma}$ subunits from $G_{i/o}$ proteins often employ transactivated receptor tyrosine kinases or Src family nonreceptor tyrosine kinases to trigger Ras-dependent ERK1/2 activation via the Grb2-mSos Ras activation complex. G_s proteins exert complex effects. Stimulation of adenylyl cyclase (AC) activates PKA, which can directly inhibit cRaf-1, but at the same time generates cAMP, which, along with PKA, activates B-Raf through Epac-Rap1 small G protein pathway. Arrestins sit at the center of the nexus. Arrestin-mediated desensitization of GRK-phosphorylated GPCRs terminates G protein signaling. At the

B-Raf is activated both by PKA-dependent phosphorylation of the Ras-family GTPase, Rap-1 (Vossler et al. 1997), and by cAMP binding to the Rap-1 guanine nucleotide exchange factor, Epac (DeRooij et al. 1998), permitting direct cAMP-mediated activation of a B-Raf–MEK1/2–ERK1/2 cascade. Pertussis toxin-sensitive G protein-dependent activation of ERK1/2 by $G_{i/o}$-coupled GPCRs is mediated primarily by Gβγ subunits and typically involves activation of receptor or non-receptor tyrosine kinases leading to Ras-dependent activation of c-Raf1 (van Biesen et al. 1995; Luttrell et al. 1996, 1997).

Arrestins are negative regulators of G protein signaling. Because ERK1/2 is rapidly inactivated by dephosphorylation, the time course of ERK1/2 activation by G protein-regulated effectors parallels heterotrimeric G protein activity, and arrestin-dependent GPCR desensitization plays an important negative regulatory role by limiting the duration of G protein-mediated ERK1/2 activation. In arrestin2/3 null murine embryo fibroblasts (MEFs), ERK1/2 activation by $G_{i/o}$-coupled lysophosphatidic acid (LPA) receptors results primarily from $G_{i/o}$-dependent transactivation of epidermal growth factor (EGF) receptors (Gesty-Palmer et al. 2005). Because LPA receptor desensitization is impaired by the absence of arrestins, the EGF receptor-dependent ERK1/2 signal in arrestin2/3 null MEFs is persistent, lasting for several hours in the continued presence of LPA. Reintroducing arrestin3, which restores desensitization, makes the transactivation-dependent signal transient, such that it contributes significantly to ERK1/2 activation only during the first few minutes of stimulation.

But arrestins perform dual roles. Thus, ERK1/2 activity does not cease as the receptor switches from a G protein-coupled to an arrestin-coupled signaling mode. The contribution of G protein-dependent and arrestin-dependent signals to angiotensin AT_{1A} receptor-stimulated ERK1/2 activation has been elegantly dissected using isoform-selective arrestin RNA interference, pharmacologic inhibitors, G protein-uncoupled receptor mutants, and arrestin pathway-selective ligands (Wei et al. 2003; Ahn et al. 2004a). When arrestin3 expression in HEK293 cells is downregulated, AT_{1A} receptor-stimulated ERK1/2 activation becomes transient and sensitive to PKC inhibition, indicating that it is mediated by a $G_{q/11}$–PLCβ–PKC pathway. In a reciprocal manner, inhibiting PKC in the presence of arrestin3 blocks the initial spike in ERK1/2 activity, but does not prevent the persistent late-phase response. Exposing the G protein-uncoupled DRY-AAY AT_{1A} receptor mutant to

Fig. 1 (continued) same time, arrestin2 (Arr2) and arrestin3 (Arr3) function as scaffolds, activating a GPCR-bound pool of ERK1/2. The mechanism of ERK1/2 activation affects its function by favoring phosphorylation of different targets. In general, ERK1/2 activated via G protein-mediated pathways is free to translocate to the cell nucleus and regulate gene transcription and cell cycle progression by phosphorylating transcription factors, like Elk1. Conversely, arrestin-activated ERK1/2 is confined to the cytosol where it phosphorylates multiple targets, including arrestin2, p90RSK, Mnk1, and eIF4E, and regulates non-transcriptional processes like GPCR endocytosis, cytoskeletal rearrangement/chemotaxis, and protein translation

angiotensin II, or the wild-type AT_{1A} receptor to [Sar^1-Ile^4-Ile^8]-AngII, generates only the sustained signal, which is insensitive to PKC inhibition and abolished by RNA interference targeting arrestin3. Studies performed on the β_2 adrenergic and type 1 parathyroid hormone (PTH) receptors have produced analogous results (Shenoy et al. 2006; Gesty-Palmer et al. 2006). Conversely, reintroducing arrestin3 into arrestin2/3 null MEFs confers a long lasting EGF receptor-independent ERK1/2 signal that presumably reflects restoration of the arrestin pathway (Gesty-Palmer et al. 2005).

Besides dictating the kinetics of ERK1/2 activation, arrestin-dependent signalsome formation affects the subcellular distribution of active ERK1/2. In general, ERK1/2 activated by classical receptor tyrosine kinases and heterotrimeric G protein-mediated pathways is free to translocate to the cell nucleus, where it gains access to nuclear transcription factors like Elk-1. But ERK1/2 activated by arrestin scaffolds remains part of the signalsome, at least when activated by "class B" GPCRs that form stable receptor-arrestin complexes (Oakley et al. 2000; Tohgo et al. 2003). Because the GPCR-arrestin complex is stable, activated ERK1/2 is instead targeted to the plasma membrane and early endosomes (DeFea et al. 2000a; Luttrell et al. 2001). In fact, an estimated 75–80 % of the active ERK1/2 produced in response to short-term stimulation of PAR2 is associated with the GPCR-arrestin signalsome (DeFea et al. 2000a). As a result, nuclear translocation of active ERK1/2 is retarded, and its kinase activity is directed away from nuclear, and toward cytosolic, targets (Fig. 1).

2.4 Functionally Distinct ERK Pools

The complex web of GPCR-derived signals that converge on ERK1/2 introduces a level of temporal and spatial control that ultimately defines ERK1/2 function. Arrestins are central players in the process. Because they control the balance between G protein-mediated signals that direct ERK1/2 toward the cell nucleus and signalsome-mediated responses that produce sustained cytosolic ERK1/2 activity, arrestins control the access to substrates (Stork 2002; Luttrell 2003).

2.4.1 Transcriptional Control and Cell Proliferation

The impact of arrestins on the balance between nuclear and cytosolic ERK1/2 signaling has been demonstrated for several GPCRs. For example, wild-type PAR2 predominantly utilizes an arrestin-dependent pathway to activate ERK1/2 (Defea et al. 2000a). As a result, the active ERK1/2 is excluded from the nucleus and does not stimulate proliferation. In contrast, a C-terminal phosphorylation site mutant of PAR2 that does not bind arrestins or internalize activates ERK1/2 via a G protein-dependent pathway that promotes its nuclear translocation and elicits a proliferative response. The angiotensin AT_{1A} behaves similarly. Wild-type AT_{1A}

receptors activate ERK1/2 using both G protein-mediated and arrestin-scaffolded pathways, increasing both cytosolic and nuclear ERK1/2 (Tohgo et al. 2002; Ahn et al. 2004a), whereas a G protein-uncoupled DRY-AAY AT_{1A} receptor mutant, which only utilizes the arrestin pathway, only activates cytosolic ERK1/2 and fails to elicit a detectable transcriptional response (Lee et al. 2008).

The question of whether arrestins ever stimulate ERK1/2-dependent transcription is less clear, especially in the case of "class A" GPCRs that dissociate from arrestins upon internalization (Oakley et al. 2000). Class A receptors, like the β_2 adrenergic and LPA receptors, appear to use arrestin scaffolds to activate ERK1/2 (Shenoy et al. 2006; Gesty-Palmer et al. 2005), but the transient nature of the receptor-arrestin interaction does not support endosomal ERK1/2 targeting. Domain swapping experiments between the class A β_2 adrenergic receptor and the class B V_2 vasopressin receptor suggest that the stability of the receptor-arrestin interaction impacts ERK1/2 function (Tohgo et al. 2003). Exchanging the V_2 receptor C terminus for that of the β_2 receptor, which converts the V_2 receptor from stable to transient arrestin binding, increases the proportion of ERK1/2 that enters the cell nucleus and permits the chimeric receptor to stimulate cell proliferation. The opposite effect is obtained when the V_2 receptor C terminus is appended to the β_2 receptor. Similarly, although most of the early LPA-stimulated transcriptional responses in arrestin2/3 null MEFs are driven by G protein-dependent EGF receptor transactivation, reintroducing arrestin3 into the null background permits LPA to elicit ERK1/2-dependent responses that do not require the EGF receptor, suggesting that dissociation of the LPA receptor–arrestin complex upon internalization permits ERK1/2 activated by the arrestin pathway to reach the nucleus (Gesty-Palmer et al. 2005).

2.4.2 Plasma Membrane and Cytosolic Targets

The ligand-induced assembly of stable GPCR–arrestin–ERK1/2 signalsomes, while retarding ERK1/2 nuclear translocation and transcription, preferentially targets ERK1/2 toward membrane and cytosolic substrates. Three extranuclear processes in which arrestin-bound ERK1/2 may play a regulatory role are GPCR desensitization, cytoskeletal rearrangement and chemotaxis, and activation of protein translation.

Within the signalsome, activated ERK1/2 modulates receptor desensitization by phosphorylating GRKs and arrestins. Arrestin-dependent phosphorylation of GRK2 Ser^{670} by ERK1/2 is agonist dependent, enhanced by prior c-Src phosphorylation, and accelerates GRK2 turnover (Elorza et al. 2003). ERK1/2 also phosphorylates Ser^{412} in the C terminus of arrestin2 (Lin et al. 1997, 1999). Free cytosolic arrestin2 is almost stoichiometrically phosphorylated on Ser^{412}, and it must be dephosphorylated upon receptor binding to engage clathrin and support receptor internalization. This dephosphorylation step apparently involves another arrestin2-bound effector, protein phosphatase 2A (Hupfeld et al. 2005). Rephosphorylation of Ser^{412} by ERK1/2 either provides negative feedback regulation of receptor endocytosis or facilitates receptor

internalization by promoting the dissociation of arrestin and clathrin, allowing the receptor to exit clathrin-coated vesicles. Ser^{412} phosphorylation may also disrupt the arrestin2-Src interaction, possibly regulating Src-dependent signals emanating from the signalsome (Luttrell et al. 1999).

Arrestins play a key role in GPCR-mediated chemotaxis, the process whereby migrating cells follow a concentration gradient to its source. Chemoattractant receptor activation induces actin cytoskeletal rearrangement, leading to formation of a dominant pseudopodium at the leading edge that protrudes forward driven by F-actin polymerization and actin–myosin contraction forces (Machesky 1997; Brahmbhatt and Klemke 2003). Splenocytes derived from arrestin3 null mice exhibit strikingly impaired chemotactic responses to stromal cell-derived factor-1, CXCL12 (Fong et al. 2002). While impaired gradient sensing due to the loss of arrestin-mediated desensitization is a contributing factor (Aragay et al. 1998), substantial evidence indicates that arrestin-dependent recruitment of ERK1/2 to chemoattractant receptors at the leading edge is required for GPCR-mediated cortical actin assembly and chemotaxis (Ge et al. 2003, 2004; Barnes et al. 2005; Hunton et al. 2005). PAR2-induced chemotaxis in MDA breast cancer cells requires both arrestin2 and 3 (Ge et al. 2004). During chemotaxis, a PAR2–arrestin–ERK1/2 complex localizes to the leading edge that activates actin cytoskeleton reorganization (Ge et al. 2003). In addition, arrestins scaffold a complex containing the actin filament-severing protein, cofilin, LIM kinase, and the cofilin-specific phosphatase, chronophin, which is required for the dephosphorylation and activation of cofilin (Zoudilova et al. 2007; *see* chapter "Molecular Mechanisms Underlying Beta-Arrestin-Dependent Chemotaxis and Actin-Cytoskeletal Reorganization"). Arrestin-bound cofilin generates the free barbed ends on actin filaments that permit filament extension. In angiotensin AT_{1A} receptor-expressing HEK 293 cells, angiotensin II, as well as the arrestin pathway-selective agonist, $[Sar^{1}\text{-}Ile^{4}\text{-}Ile^{8}]$-AngII, promote chemotaxis through an arrestin3-dependent mechanism that is independent of G protein activity (Hunton et al. 2005). Analogous results have been obtained in primary murine preosteoblasts, where the arrestin pathway selective type 1 PTH receptor agonist, [D-Trp12,Tyr34]-bPTH(7-34), stimulates migration in wild type, but not arrestin3 null, preosteoblasts (Gesty-Palmer et al. 2013). Arrestins also affect cell shape change by interacting with the actin-bundling protein, filamin A. Assembly of an AT_{1A} receptor–arrestin–ERK1/2–filamin A complex is required for the formation of membrane ruffles in Hep2 cells (Scott et al. 2006).

Arrestin-dependent cytosolic targeting of ERK1/2 also appears to regulate protein translation. Cytosolic ERK1/2 substrates include the ribosomal S6 kinase, p90RSK (Aplin et al. 2007), and MAP kinase-interacting kinase 1 (Mnk1), a regulator of the ribosomal protein translation initiation complex (DeWire et al. 2008). ERK1/2 phosphorylation of p90RSK is activated by a mutant angiotensin AT_{1A} receptor with a deletion in its second intracellular loop that inhibits G protein coupling (Seta et al. 2002). Using RNA interference to downregulate arrestin3, it has been shown that arrestin-dependent ERK1/2 activation by the AT_{1A} receptor mediates phosphorylation of Mnk1 and eukaryotic translation initiation factor 4E (eIF4E), increasing rates of mRNA translation (DeWire et al. 2008).

2.5 *Receptors, GRKs, and Posttranslational Modifications*

While the basic model of arrestin-dependent scaffolding of the ERK1/2 cascade appears to hold across a wide range of GPCRs, numerous factors introduce variations on the theme that add selectivity or tailor the response to the specific receptor and cellular context. Among these are the influence of receptor structure and arrestin isoform selectivity, the role of GRKs in specifying arrestin conformation, and the effects of arrestin posttranslational modifications.

2.5.1 Arrestin Isoform Selectivity

In cells, arrestins 1, 2, and 3, but not arrestin4, are able to bind ERK1/2 and redistribute it to microtubules (Hanson et al. 2007), but the capacity of different arrestin isoforms to support GPCR-catalyzed ERK1/2 activation varies between receptors. The most obvious difference is between class A and class B receptors and reflects the arrestin binding preference of the receptor. Most GPCRs can be separated into one of two classes based on their affinity for the two nonvisual arrestin isoforms and the longevity of the receptor–arrestin interaction (Oakley et al. 2000). Class A receptors have higher affinity for arrestin3 than arrestin2 and form transient receptor–arrestin complexes that dissociate soon after the receptor internalizes. Such receptors are rapidly resensitized and recycled back to the plasma membrane. Class B receptors have equivalent affinities for arrestins 2 and 3 and form stable receptor–arrestin complexes that remain intact as the receptor undergoes endosomal sorting. As previously discussed, although both class A and class B receptors are capable of mediating arrestin-dependent ERK1/2 activation, class B receptors, like the PAR2 and angiotensin AT_{1A} receptors, are more effective at targeting active ERK1/2 to endosomes (Defea et al. 2000a; Luttrell et al. 2001). In contrast, class A receptors, like the β_2 adrenergic and LPA receptors, which fail to traffic ERK1/2 to endosomes, may use arrestin scaffolds to generate a transcriptionally competent ERK1/2 pool (Shenoy et al. 2006; Gesty-Palmer et al. 2005).

Curiously, the contribution of arrestins 2 and 3 to ERK1/2 signaling by class B receptors varies. In the case of angiotensin AT_{1A} receptors expressed in HEK293 cells, arrestin2 and 3 perform opposing functions (Ahn et al. 2004b; Lee et al. 2008). Whereas downregulating endogenous arrestin3 expression by RNA interference inhibits wild-type AT_{1A} receptor ERK1/2 activation by about 50 %, and abrogates ERK1/2 activation by a G protein-uncoupled DRY-AAY AT_{1A} receptor mutant or in response to [Sar^1-Ile^4-Ile^8]-AngII, silencing arrestin2 expression paradoxically enhances the ERK1/2 signal. This has led to the hypothesis that, with respect to ERK1/2 activation, arrestin3 is the "signaling" arrestin isoform, while arrestin2 functions only in desensitization (Ahn et al. 2004b). The vasopressin V_2 receptor exhibits similar reciprocal regulation of ERK1/2 activity (Ren et al. 2005). But the functional dichotomy between arrestin2 and 3 does not hold

for all class B receptors. For example, PAR1 exhibits reciprocal regulation of ERK1/2, but in a manner opposite that of the AT_{1A} receptor. In this case, silencing arrestin2 results decreased ERK1/2 activation while silencing of arrestin3 increases thrombin-stimulated ERK1/2 activation (Kuo et al. 2006). The type 1 PTH receptor exhibits yet another pattern. With this class B receptor, arrestin-dependent ERK1/2 activation is inhibited when either isoform is downregulated, suggesting that both are required to assemble functional signalsomes (Gesty-Palmer et al. 2006, 2009). G protein-independent ERK1/2 activation by the β_2 adrenergic receptor, despite its class A preference of arrestin3, shows a similar codependent pattern of arrestin-mediated ERK1/2 activation (Shenoy et al. 2006).

2.5.2 GRKs and "Bar Codes"

The observation that a single arrestin isoform can promote ERK1/2 activation by one receptor while antagonizing activation by another suggests that arrestins are not inherently specialized, but instead can adopt distinct "signaling" or "desensitizing" conformations (Xiao et al. 2004; Nobles et al. 2007). Experimental evidence suggests that the pattern of GRK phosphorylation on the GPCR C terminus affects arrestin conformation and, hence, function, even for a single receptor. Data obtained through isoform-selective silencing of GRK2, 3, 5, and 6 in HEK293 cells suggests that GRK2 and GRK3 phosphorylation of the angiotensin AT_{1A} receptor favors arrestin-dependent desensitization, while GRK5 and GRK6 appear to be exclusively responsible for initiating arrestin-dependent ERK1/2 activation (Kim et al. 2005). Identical results have been reported for the vasopressin V_2 receptor (Ren et al. 2005). Computational modeling based on AT_{1A} receptor signaling and desensitization in HEK293 cells and primary vascular smooth muscle predicts that in addition to its critical role in desensitization of G protein signaling, GRK2 exerts a negative effect on arrestin-dependent signaling through competition with GRK5 and 6 for receptor phosphorylation (Heitzler et al. 2012).

Quantitative mass spectroscopic analysis of phosphorylation sites in the β_2 adrenergic C terminus suggests that GRK2 and GRK6 preferentially phosphorylate different sites and that only the GRK6-induced pattern of phosphorylation correlates with the ability of isoproterenol or the arrestin pathway-selective inverse agonist, carvedilol, to support arrestin-dependent ERK1/2 activation. Isoform-selective knockdown of GRK2 or GRK6 changes the amplitude and direction of conformational shifts in arrestin3 monitored by bioluminescence resonance energy transfer (BRET) using an intramolecular arrestin3 BRET reporter. This has led to the hypothesis that different GRKs establish a phosphorylation "barcode" on the receptor C terminus that imparts distinct arrestin3 conformations to regulate its functional activity (Nobles et al. 2011).

2.5.3 Posttranslational Modifications

Arrestins undergo numerous regulated posttranslational modifications, among them phosphorylation (Lin et al. 1999, 2002), ubiquitination (Shenoy 2007; *see* chapter "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications"), and S-nitrosylation (Ozawa et al. 2008), that impact their function in GPCR desensitization and signaling. Phosphorylation of arrestin2 on Ser^{412} (Lin et al. 1999), and of arrestin3 on Thr^{383} and Ser^{361} (Lin et al. 2002), reportedly regulates their interaction with clathrin and subsequent internalization of receptors. As previously discussed, arrestin-bound ERK1/2 may modulate GPCR endocytosis by rephosphorylating arrestin2 Ser^{412}. Another putatively arrestin-regulated kinase, casein kinase II (Xiao et al. 2010; Kendall et al. 2011), may perform the analogous function by phosphorylating Thr^{383} of arrestin3.

E3 ubiquitin ligases catalyze the transfer of ubiquitin to the ε-amino group of lysine residues in substrate proteins. Arrestins interact with at least four different E3 ubiquitin ligases, Mdm2, AIP4, Nedd4, and TRAF6 (Shenoy et al. 2001, 2008; Wang et al. 2006; Bhandari et al. 2007), as well as the deubiquitinase, USP33 (Shenoy et al. 2009). The reversible ubiquitination of arrestin3 affects the stability of the GPCR–arrestin signalsome complex and the characteristics of receptor desensitization, internalization, and trafficking. Mdm2 and USP33 function reciprocally in regulating internalization of the β_2 adrenergic receptor (Shenoy et al. 2009). Both enzymes bind arrestin3. Mdm2-dependent ubiquitination stabilizes the receptor–arrestin complex, since genetic ablation of Mdm2 blocks β_2 receptor endocytosis (Shenoy et al. 2001). Although a lysine-less mutant of arrestin3 retains the ability to bind the β_2 receptor in vitro, and ERK1/2 pathway components in cells, it binds poorly to clathrin and is unable to assemble an ERK1/2 signalsome. On the other hand, expression of a stably ubiquitinated arrestin3-ubiquitin chimera not only stabilizes the ERK signalsome but also supports endosomal targeting, producing a class B receptor-like pattern of endosomal ERK1/2 targeting (Shenoy and Lefkowitz 2003; Shenoy et al. 2007).

Analogous studies with the angiotensin AT_{1A} receptor support the role of arrestin ubiquitination in controlling signalsome function (Shenoy and Lefkowitz 2005). Unlike the β_2 adrenergic receptor, which is rapidly deubiquitinated by USP33 following internalization, arrestin3 remains stably ubiquitinated on Lys 11 and 12 when bound to the AT_{1A} receptor. Expression of a $K^{11/12}R$ arrestin3 mutant reverses the pattern of arrestin binding, such that the AT_{1A} receptor adopts the β_2 receptor class A pattern of transient arrestin binding and impaired ERK1/2 activation. Conversely, an arrestin3($K^{11/12}R$)-ubiquitin chimera restores endosomal trafficking of arrestin-bound AT_{1A} receptors and endosomal targeting of ERK1/2 signalsomes.

2.6 Ligand-Dependent Stimulus Trafficking

Recently, considerable excitement has arisen over the finding that in addition to intracellular factors, GPCR ligand structure is a determinant of arrestin function (Kenakin and Miller 2010; Luttrell and Kenakin 2011; Whalen et al. 2011). Classical receptor theory is grounded in the premise that GPCRs exist in equilibrium between single inactive and active states and that the intrinsic efficacy of a ligand is a reflection of its ability to selectively bind and stabilize the active or inactive state (Kenakin 1996; Samama et al. 1993). While these models adequately describe the behavior of conventional agonist, antagonist, and inverse agonist ligands, they cannot accommodate the phenomena of reversal of potency, where the rank order of potency for a series of ligands acting on a common receptor differs depending on the response being measured, or reversal of efficacy, where a ligand exerts opposing effects on receptor coupling to different effectors. Such behavior can only be modeled based on the assumption that the receptor can exist in more than one "active" conformation. The concept of functional selectivity, originally developed in the 1990s, proposes that GPCRs adopt multiple "active" conformations that couple the receptor to downstream effectors with different efficiency and that chemically distinct ligands can affect the conformational equilibrium of the receptor in ways that "bias" downstream coupling compared to the native ligand (Christopoulos and Kenakin 2002) (see chapter "Quantifying Biased β-Arrestin Signaling").

The observation that arrestin-dependent signaling is not necessarily dependent upon prior G protein activation quickly lead to the discovery that some ligands that function as antagonists or inverse agonists of receptor-G protein coupling were, in fact, arrestin pathway-selective "biased" agonists. The peptide angiotensin II analogue, [Sar^1-Ile^4-Ile^8]-AngII, antagonizes $G_{q/11}$ coupling but promotes GRK phosphorylation, arrestin recruitment, and receptor endocytosis (Holloway et al. 2002). In HEK293 and primary vascular smooth muscle cells, [$Sar^1Ile^4Ile^8$]-AngII promotes sustained arrestin-dependent ERK1/2 activation in the absence of $G_{q/11}$/PLCβ/PKC pathway activation (Wei et al. 2003; Miura et al. 2004). In primary cardiomyocytes, SII activates cytosolic, but not nuclear, ERK1/2, leading to activation of p90RSK, but not the nuclear transcription factor Elk1 (Aplin et al. 2007). A number of β_2 adrenergic receptor ligands have also been classified, or reclassified, as arrestin-selective agonists (Drake et al. 2008). Carvedilol is a nonselective $\beta_{1/2}$ and α_1 adrenergic receptor antagonist that demonstrates inverse agonist activity toward β_2 receptor-G_s coupling, but stimulates GRK-mediated phosphorylation of the receptor C terminus and arrestin-dependent ERK1/2 activation (Wisler et al. 2007). Similarly, the nonselective $\beta_{1/2}$ receptor antagonist, propranolol, and the β_2 receptor-selective agent, ICI 118551, have inverse agonist effects on cAMP production but support arrestin-dependent ERK1/2 activation (Azzi et al. 2003). Another arrestin pathway-selective agonist exhibiting reversal of efficacy is [D-Trp^{12}-Tyr^{34}]-bPTH(7-34), an inverse agonist of type 1 PTH

receptor-G_s coupling that acts as an arrestin-dependent agonist for ERK1/2 activation, antiapoptotic signaling, and cell migration, in HEK293 cells and primary murine osteoblasts (Gesty-Palmer et al. 2006, 2009; Appleton et al. 2013).

Given the expanding number of GPCRs for which arrestin-biased ligands have been characterized, it is increasingly likely that most GPCRs can adopt distinct G protein- and arrestin-coupled signaling states. Indeed, signaling bias also exists among naturally occurring GPCR ligands. The two endogenous ligands of the CCR7 chemokine receptor, CCL19 and CCL21, have similar receptor binding affinities in CCR7-transfected HEK 293 cells, and both induce chemotaxis with equal potency (Sullivan et al. 1999). However, CCL19 induces G_i activation, receptor phosphorylation, desensitization, and arrestin-dependent ERK1/2 activation, whereas CCL21 activates G_i without leading to receptor desensitization (Kohout et al. 2004).

The physical basis ligand-dependent "bias" of arrestin function appears related to their ability to induce different arrestin conformations upon receptor binding. Data obtained using an intramolecular BRET probe of arrestin3 conformation indicate that conventional and arrestin-biased ligands cause different conformational shifts in arrestin (Shukla et al. 2008). For three different GPCRs, the angiotensin AT_{1A}, β_2 adrenergic, and type 1 PTH receptors, conventional agonist binding increased BRET between donor and acceptor fluorophores attached to the arrestin3 N- and C-termini, while in each case an arrestin-selective agonist produced a small decrease. Such data suggest that by stabilizing different active receptor states, ligands can engender arrestin conformations that may serve as a "signature" of their downstream functionality, potentially biasing not only whether the arrestin binds the receptor, but also what it does once bound. Data obtained with CCL19 and CCL21 suggest that differences in the efficiency with which ligands promote GRK2/3 versus GRK5/6 phosphorylation may underlie differences in arrestin recruitment (Zidar et al. 2009), consistent with the "barcode" hypothesis that the desensitizing and signaling functions of arrestins are encoded by GRK-specific patterns of receptor phosphorylation (Nobles et al. 2011).

3 Arrestin-Dependent Activation of Src Family Kinases

The first arrestin-dependent signaling event to be discovered was the agonist-induced recruitment of the nonreceptor tyrosine kinase, c-Src, to β_2 adrenergic receptors (Luttrell et al. 1999). Early studies of GPCR-stimulated ERK1/2 activation had suggested a paradoxical link between GPCR endocytosis and the positive regulation of Ras-dependent signaling (Daaka et al. 1998; Ignatova et al. 1999). It was likewise clear that Src family kinases could be activated by a number of GPCRs (Luttrell and Luttrell 2004) and that Src activity was involved in GPCR cross talk with receptor tyrosine kinases upstream of Ras activation (Luttrell et al. 1996, 1997). The observation that c-Src bound directly to arrestin2 and redistributed

along with it to the GPCR on the plasma membrane provided one mechanism for GPCR regulation of growth factor signaling pathways and led to the concept of arrestin-based GPCR "signalsomes" (Luttrell et al. 1999).

3.1 Src Family Nonreceptor Tyrosine Kinases

c-Src is the prototypic member of a family of nine nonreceptor tyrosine kinases that participate in the control of cell proliferation, survival, adhesion, cytoskeletal rearrangement, and vesicle trafficking by cell surface receptors. Of these, three, Src, Fyn, and Yes, are widely expressed, while other family members, including Fgr, Fyn, Hck, Lck, and Lyn, are largely confined to cells of hematopoietic lineage (Erpel and Courtneidge 1995). The Src family kinases share a common domain architecture, consisting of a variable N terminus followed by Src homology (SH)3 and SH2 domains, the kinase (SH1) domain, and a C-terminal regulatory domain. The kinase domain of Src family kinases bears homology to the catalytic domains of other tyrosine kinases, including the *c-Abl* and *c-Fps* families of cytosolic tyrosine kinases and the EGF, insulin, and platelet-derived growth factor families of receptor tyrosine kinases (Superti-Furga and Courtneidge 1995). The SH2 domain, which recognizes specific phospho-tyrosine motifs, and SH3 domain, which binds proline-rich, Pro-X-X-Pro, motifs, regulate Src activity and control its interactions with binding partners. Src activity is tightly controlled in cells through an auto-inhibitory interaction between a conserved tyrosine in the C terminus, Tyr^{530} in mammalian c-Src, and the SH2 domain, which precludes substrate access to the SH1 domain. Another nonreceptor tyrosine kinase, c-Src Kinase (Csk), maintains Tyr^{530} in the phosphorylated state. Src activation involves dephosphorylation of Tyr^{530} followed by autophosphorylation of a Tyr residue in the active site, Tyr^{419} in mammalian c-Src, which fully activates the kinase. Indeed, truncation of the C terminus, as occurs in the oncogenic Rous sarcoma virus gene product, v-Src, or experimental mutation of the C-terminal Tyr residue to Phe, produces a constitutively active kinase that is fully transforming. Once the dephosphorylated C terminus is released, the Src SH2 domain is free to engage phospho-tyrosine motifs in binding partners, such as the EGF receptor (Liu and Pawson 1994).

As cytosolic proteins with no direct means of detecting changes in the extracellular milieu, the function of Src family kinases is regulated by their recruitment to cell surface receptors through their SH2 and SH3 domains. The result is that Src signaling occurs in the context of multiprotein complexes in which an activated receptor provides a scaffold whose role is to recruit an assortment of enzymatic effectors. Native Src family kinases are activated by many growth factor receptor tyrosine kinases and are key components of the signaling complexes assembled following receptor autophosphorylation. In addition, the interaction of Src family

kinases with focal adhesion kinases, such as p125FAK and Pyk2, targets them to focal adhesions, where they play an essential role in the cytoskeletal changes and signaling events that result from activation of integrins (Luttrell and Luttrell 2004).

3.2 Binding and Scaffolding of Src Family Kinases

Arrestins 1, 2, and 3 bind Src family kinases and recruit them to activated GPCRs. As with the components of the ERK1/2 cascade, the Src binding sites on arrestins have not been mapped precisely and appear to involve multiple points of contact. From co-immunoprecipitation studies, c-Src appears to interact primarily with the N-terminal domain of arrestin2 (Luttrell et al. 1999), which is proline rich and contains three potential SH3 domain-binding Pro-X-X-Pro motifs. A mutated arrestin2, [Pro^{91}Gly–Pro^{121}Glu]-arrestin2, in which two of the three Pro-X-X-Pro motifs are disrupted, exhibits impaired c-Src binding and functions as a dominant negative inhibitor of β_2 adrenergic receptor-mediated ERK1/2 activation. In vitro, arrestin2 binds directly to c-Src SH3, but not SH2, domain glutathione S-transferase fusion proteins. However, the c-Src SH1 domain also contributes to arrestin2 binding, since its deletion markedly reduces c-Src binding to full-length arrestin2, while the isolated c-Src SH1 domain retains strong binding even without the SH2 and SH3 domains (Miller et al. 2001). The arrestin2-Src SH1 domain interaction is independent of kinase activity, since catalytically inactive Lys^{298}Met mutants of both full-length c-Src and the isolated SH1 domain retain strong arrestin1 binding (Luttrell et al. 1999; Miller et al. 2000). Arrestin1 also binds c-Src, but the interaction is different. Photobleaching of rod outer segments leads to association of active c-Src and arrestin1 with rhodopsin (Ghalayini et al. 2002). Arrestin1 has only a single Pro-*X*-*X*-Pro motif in the N-terminal domain, and bleached rhodopsin and arrestin1 interact with c-Src SH2 domain and SH2-SH3 domain glutathione S-transferase proteins equivalently, suggesting that in this case the c-Src SH2 domain mediates the interaction.

Unlike the components of the ERK1/2 cascade (*see* Sect. 2.2), the affinity of c-Src for mutationally stabilized free, receptor- and microtubule-bound conformations of arrestins has not been determined. Co-immunoprecipitation studies using β_2 adrenergic receptors suggest that both the association of c-Src with arrestin2 and the arrestin-dependent recruitment of c-Src to the receptor are agonist dependent (Luttrell et al. 1999). Similarly, the neurokinin NK1 receptor forms a receptor-arrestin2-Src complex in response to substance P stimulation (DeFea et al. 2000b). Gel filtration of NK1 receptor complexes from stimulated cells reveals a larger-than-expected complex size of ~300 kDa, suggesting the ligand-dependent assembly of NK1 receptor-arrestin2-Src complex containing additional proteins. Although the c-Src associated with bleached rhodopsin-bound arrestin1 and β_2 receptor-bound arrestin2 is Tyr^{530} dephosphorylated, and therefore presumably active, c-Src activation is not a prerequisite for arrestin binding, since both catalytically inactive Lys^{298}Met and constitutively active Tyr^{530}Phe mutants of c-Src

bind equivalently (Ghalayini et al. 2002; Luttrell et al. 1999). Nor is internalization of the receptor-arrestin complex required for Src binding, since isoproterenol-stimulated recruitment of active c-Src to β_2 adrenergic receptor-arrestin complexes at the plasma membrane can be observed following antibody cross-linking of receptors, which causes them to be retained at the cell surface (Luttrell et al. 1999). On the other hand, the c-Src binding to arrestin2 may be regulated by arrestin2 Ser^{412} phosphorylation, since a non-phosphorylatable Ser^{412}Ala mutant of arrestin2 retains wild-type binding to c-Src, while a phospho-mimetic Ser^{412}Asp is impaired (Luttrell et al. 1999). Ligand-induced dephosphorylation of Ser^{412}, which occurs upon arrestin2 binding to the β_2 receptor, may thus provide a mechanism for regulating complex assembly (Lin et al. 2002).

3.3 Functions of Arrestin-Bound Src Family Kinases

Like the MAPKs, Src family kinases are functionally pleiotropic. Although most intensively studied because of their oncogenic potential and involvement in Ras-dependent signaling by growth factor receptor tyrosine kinases, Src family kinases play regulatory roles in other processes, including survival signaling, cell adhesion, cytoskeletal rearrangement, and vesicle trafficking. The mechanisms whereby GPCRs regulate Src activity are similarly diverse, involving both direct interactions between Src family kinases and G protein subunits or arrestins and indirect regulation of receptor tyrosine kinase and focal adhesion complexes by G protein-activated effectors (Luttrell and Luttrell 2004). The role of arrestins in GPCR-Src signaling appears to be analogous to its role in ERK1/2 regulation. By binding Src family kinases and targeting them to specific cellular locations, arrestins support the activation of spatially localized kinase pools that perform specific functions related to GPCR control of mitogenic signaling pathways and regulation of vesicle trafficking. Fig. 2 provides a schematic depiction of how arrestin scaffolding of Src family kinases contributes to arrestin-dependent regulation of GPCR function.

3.3.1 Receptor Cross Talk and Cell Proliferation

One prevalent mechanism for GPCR control of cell proliferation is through "transactivation" of the EGF receptor family of tyrosine kinases (Carpenter 2000). Endogenous EGF receptor ligands are synthesized as transmembrane precursors that must be proteolytically processed by an ADAM (a disintegrin and metalloprotease) family matrix metalloproteases to generate the soluble growth factor. Upon ligand binding, monomeric EGF receptors dimerize, transphosphorylate on tyrosine residues within their intracellular domains, and recruit SH2 domain-containing adapter proteins, such as c-Src, Shc, and the Grb2-mSos complex, to assemble a mitogenic signaling complex that catalyzes Ras activation

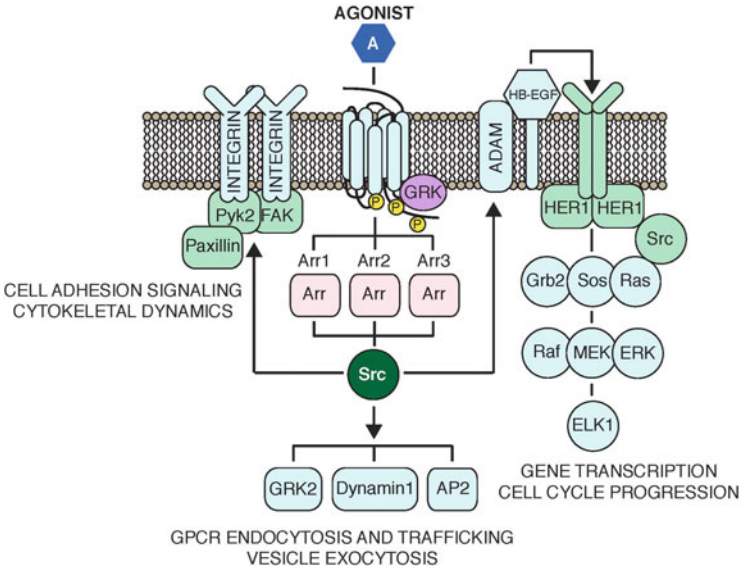

Fig. 2 Arrestin-dependent regulation of Src family kinases impacts multiple processes. Upon agonist (A) binding, arrestins 1, 2, and 3 can bind Src family kinases and recruit them into receptor-based signalsomes. Arrestin-dependent Src activation has been implicated in several GPCR signaling functions. In some cells, arrestins and Src regulate the activity of ADAM family matrix metalloproteases, leading to cleavage and release of EGF receptor ligands, like HB-EGF, and autocrine/paracrine transactivation of EGF receptors (HER1). Downstream Ras-dependent activation of the cRaf-1-MEK-ERK cascade by the Grb2-Sos complex, a process that also involves Src, generates a transcriptionally competent pool of ERK1/2 that can activate Elk1 transcription and drive cell cycle progression. Arrestin-Src complexes also regulate integrin-based focal adhesion signaling by modulating the activity of the focal adhesion kinases, p125FAK and Pyk2, and the actin-adapter protein paxillin. Focal adhesion complexes, in turn, regulate cytoskeletal dynamics and chemotactic cell migration and generate signals controlling cell proliferation and survival. In addition, arrestins direct the Src-dependent phosphorylation of proteins involved in GPCR endocytosis and vesicle trafficking, including GRK2, dynamin1, and the β2-adaptin subunit of AP-2

and Ras-dependent ERK1/2 signaling. Many GPCRs stimulate ADAM-dependent ectodomain shedding, promoting release of EGF receptor ligands like heparin-binding (HB)-EGF and activating EGF receptor pathways (Prenzel et al. 1999). The contribution of c-Src to GPCR-mediated EGF receptor transactivation was elegantly dissected using a co-culture system in which the pathway components upstream of α_{2A} adrenergic receptor-stimulated HB-EGF shedding could be distinguished from elements functioning downstream of the activated EGF receptor (Pierce et al. 2001a, b). Whereas $G_{\beta\gamma}$ subunits and matrix metalloprotease activity were required to release HB-EGF, and EGF receptor kinase activity and dynamin-dependent endocytosis were necessary for EGF receptor-dependent ERK1/2 activation, Src activity was found to be involved both upstream of HB-EGF release and downstream of the EGF receptor.

In some settings, arrestin-Src complexes perform the upstream role, linking GPCR activation to metalloprotease-dependent EGF receptor ligand shedding (Fig. 2). The luteinizing hormone receptor activates c-Fyn in an arrestin3-dependent manner (Galet and Ascoli 2008). Downregulating arrestin expression inhibits receptor-mediated activation of c-Fyn; phosphorylation of the antiapoptotic focal adhesion kinase, p125FAK; and the release of EGF-like growth factors. In primary vascular smooth muscle, [Sar^1-Ile^4-Ile^8]-AngII induces ERK1/2 activation and cell proliferation by promoting angiotensin AT_{1A} receptor-dependent EGF receptor transactivation (Miura et al. 2004). Both angiotensin II and [Sar^1-Ile^4-Ile^8]-AngII stimulate Src-dependent EGF receptor phosphorylation on Tyr^{845}, an effect that is lost when arrestin3 is downregulated by RNA interference (Kim et al. 2009). In HEK293 cells expressing β_1 adrenergic receptors, EGF receptor transactivation and ERK1/2 activation are inhibited by downregulating arrestin2 or 3, or GRK5 or 6; inhibiting Src or MMP activity; or exposing to a heparin-binding-EGF neutralizing antibody, suggesting that β_1 receptor-mediated EGF receptor transactivation is arrestin dependent (Noma et al. 2007). Consistent with this, a mutant β1 receptor lacking the GRK phosphorylation sites in its C-terminal tail (-GRKβ1), which cannot undergo arrestin-dependent desensitization, fails to transactivate EGF receptors despite exaggerated G protein activation. It is important to mention, however, that arrestins are clearly not the sole mediators of GPCR-stimulated ectodomain shedding, since ERK1/2 activation by LPA receptors in arrestin2/3 null MEFs is almost entirely due to G protein-dependent transactivation of EGF receptors (Gesty-Palmer et al. 2005).

Unlike the direct arrestin-dependent scaffolding ERK1/2, in which signalsome-bound ERK1/2 is transcriptionally repressed (*see* Sect. 2.4.1), signaling via arrestin-Src can stimulate ERK1/2-dependent transcription and promote cell proliferation and survival. Transgenic mice expressing the -GRKβ1 receptor in cardiomyocytes develop more severe dilated cardiomyopathy in response to chronic isoproterenol stimulation. In this model, inhibiting EGF receptors worsens the cardiomyopathy, suggesting that arrestin-dependent EGF receptor transactivation exerts pro-survival effects in the heart (Noma et al. 2007). Likewise, neointimal hyperplasia following carotid endothelial injury is diminished in arrestin3 null mice. Loss of arrestin3 is associated with decreased GPCR-stimulated ERK1/2 activation, migration, and proliferation of vascular smooth muscle cells, consistent with a stimulatory role for arrestin3 in the proliferative response (Kim et al. 2008). In this system, knockout of arrestin2 has the opposite effect, suggesting that arrestin2 and 3 play opposing roles in regulation of vascular smooth muscle proliferation. As with arrestin-ERK1/2, however, the functional specialization of arrestin2 and 3 varies. The glucagon-like peptide (GLP-1) receptor promotes Src-dependent proliferation of pancreatic β cells through arrestin2. In these cells, knockdown of arrestin2 prevents GLP-1 receptor activation of c-Src, and cells expressing an arrestin2 mutant incapable of interacting with c-Src do not proliferate in response to GLP-1. Conversely, cells expressing an arrestin2 mutant capable of activating c-Src, but unable to promote receptor endocytosis, exhibit prolonged Src activation and an exaggerated GLP-1 response (Talbot et al. 2012).

Arrestin signaling also promotes cell proliferation in some forms of cancer. Human bladder cancer cells express high levels of both the thromboxane TP-β receptor isoform and arrestin3, and the degree of TP-β upregulation correlates with poorer prognosis (Mossa et al. 2008). TP-α and TP-β are splice variants that differ only in the C terminus, with TP-β carrying a longer tail that allows it to engage arrestin3 and undergo agonist-dependent internalization (Parent et al. 1999). Expressing TP-β in nontransformed SV-HUV urothelial cells confers agonist-dependent ERK1/2 and p125FAK phosphorylation and enhances cell proliferation, migration, and invasion in vitro, responses that are lost when arrestin3, but not arrestin2, is downregulated by RNA inference. Similarly, arrestin2-dependent activation of c-Src and EGF receptor appears to contribute to the tumor promoting effects of Prostaglandin EP2 receptors in papilloma formation (Chun et al. 2009). In non-small-cell lung cancer, arrestin2-Src also increases cell proliferation by activating an Rb-Raf-1 pathway that promotes Rb dissociation from E2F-responsive proliferative promoters, leading to increased E2F1 binding, transcription of S-phase genes, and cell cycle progression (Dasgupta et al. 2006).

Like receptor tyrosine kinases, focal adhesions serve as platforms from which GPCRs generate Src-dependent signals. Focal adhesions form when integrin heterodimers, which serve as extracellular matrix receptors, cluster at points of contact between the cell surface and specific matrix proteins. Part of this complex, p125FAK, associates with Src and the Ras activation complexes, Grb2-Sos1 and Crk-C3G, to initiate signals regulating cell proliferation and differentiation. Src is an essential component of the complex, as it binds p125FAK and phosphorylates it, creating additional phospho-tyrosine docking sites for SH2 domain-containing proteins (Haskell et al. 2001). In some cell types, a distinct FAK family kinase, Pyk2, provides a direct link between heterotrimeric G proteins and focal adhesion signaling (Lev et al. 1995). Like p125FAK, Src family kinases are recruited and activated by binding to Pyk2 (Lev et al. 1995; Dikic et al. 1996, 1998). But unlike p125FAK, Pyk2 requires both cellular adhesion and a co-stimulatory G protein-mediated calcium and PKC signal for activation (Brinson et al. 1998; Li et al. 1998).

Given that both receptor tyrosine kinases and focal adhesions engage in cross talk with GPCRs and that both support Src-dependent Ras signaling, the contribution of each to mitogenic GPCR signaling varies in a highly cell-specific manner. For example, in neuroendocrine PC-12 cells, which express Pyk2, activation of ERK1/2 by LPA and bradykinin receptors is blocked by disrupting focal adhesions but is insensitive to EGF receptor inhibition. Conversely, in Rat-1 fibroblasts, which lack Pyk2, disrupting focal adhesions has no effect on ERK1/2 activation, while inhibiting EGF receptors abolishes the response. Src activity, however, is required for both pathways (Della Rocca et al. 1999).

In some cases, GPCR-mediated regulation of focal adhesion signaling involves arrestins (Fig. 2). As mentioned above, phosphorylation of p125FAK by the luteinizing hormone receptor is dependent upon arrestin3 and c-Lyn (Galet and Ascoli 2008), and thromboxane TP-β receptor-mediated p125FAK phosphorylation involves arrestin3 (Mossa et al. 2008). CCR5 chemokine receptor-mediated

macrophage chemotaxis in response to MIP-1β requires assembly of a multiprotein complex containing arrestin2/3, the Src family kinase, Lyn, Pyk2, and p85 phosphatidylinositol 3-kinase (Cheung et al. 2009). Downregulating arrestins 2 and 3 impairs complex formation and inhibits MIP-1β-induced chemotaxis, suggesting that the arrestins play an important scaffolding role. Similarly, arrestin2, c-Src, and ERK1/2 participate in purinergic P2Y(12) receptor-mediated chemotaxis of microglia (Lee et al. 2012). Activation of P2Y(12) receptors by ADP causes arrestin2 and ERK1/2 to translocate to focal adhesions in newly forming lamellipodia. Once there, phosphorylation of the actin-regulating adapter protein, paxillin, by c-Src promotes focal adhesion assembly. Paxillin phosphorylation by ERK1/2 has the opposite effect, destabilizing the focal adhesion complex. Downregulation of arrestin2 inhibits paxillin phosphorylation and disrupts the assembly/disassembly of focal adhesions, leading to inefficient ADP-induced chemotaxis.

3.3.2 GPCR Endocytosis and Vesicle Trafficking

Several proteins involved with GPCR desensitization, endocytosis, and trafficking are also Src substrates (Fig. 2). c-Src phosphorylates GRK2 following activation of either β_2 adrenergic or CXCR4 chemokine receptors (Sarnago et al. 1999; Fan et al. 2001; Penela et al. 2001). Src phosphorylation has complex effects on GRK2 function, increasing its intrinsic kinase activity but also targeting it for rapid ubiquitination and proteasomal degradation. Expression of a [Pro^{91}Gly-Pro^{121}Glu]-arrestin2 mutant lacking the putative c-Src SH3 binding domains inhibits Src-dependent phosphorylation and degradation of GRK2, suggesting that GRK2 phosphorylation is mediated by an arrestin2-Src complex. Src may also indirectly regulate GRK2, since GRK2 is also a substrate for ERK1/2. Phosphorylation of GRK2 by ERK1/2 decreases GRK2 translocation to the membrane and reduces GRK2 activity (Pitcher et al. 1999; Elorza et al. 2000, 2003). Since c-Src is required for GPCR activation of ERK in many systems (*see* Sect. 3.3.1), this may provide another mechanism through which it participates in feedback regulation of GPCR signaling.

Arrestin-dependent recruitment of Src kinases also controls the function of two proteins that are essential for clathrin-dependent endocytosis, dynamin1 and the β2-adaptin subunit of AP-2. c-Src phosphorylates dynamin1, a large GTPase that controls the fission of nascent clathrin-coated vesicles. Phosphorylation on Tyr^{497} promotes dynamin1 self-assembly, and expression of a dynamin1 Tyr^{497}Phe mutant impairs internalization of β_2 adrenergic and M_2 muscarinic receptors (Ahn et al. 1999, 2002; Werbonat et al. 2000). Expression of a catalytically inactive c-Src SH1 domain fragment that binds tightly to arrestin2 and disrupts arrestin-Src binding inhibits GPCR-stimulated dynamin Tyr^{497} phosphorylation and endocytosis, suggesting that arrestins target c-Src to dynamin in clathrin-coated pits (Miller et al. 2000). The β2-adaptin subunit of AP-2 is another endocytic protein whose

regulation by Src is arrestin dependent (Fessart et al. 2005, 2007; Zimmerman et al. 2009). c-Src stabilizes a constitutive association between arrestin3 and β2-adaptin independent of its kinase activity. Src-mediated phosphorylation of β2-adaptin Tyr^{737} occurs in clathrin-coated pits in response to angiotensin AT_{1A}, β_2 adrenergic, V_2 vasopressin, or B_2 bradykinin receptor activation, leading to dissociation of AP-2 from the complex. If β2-adaptin phosphorylation is blocked, receptor-arrestin complexes are retained at the membrane.

Evidence also suggests that arrestin-Src complexes play a role in the exocytosis of secretory granules. In human granulocytes, activation of the CXCR-1 chemokine receptor by interleukin-8 induces the formation of complexes between arrestin2 and the Src family kinases, Hck and Fgr (Barlic et al. 2000). Granulocytes expressing the [Pro^{91}Gly-Pro^{121}Glu]-arrestin2 mutant fail to activate Hck and exhibit impaired chemokine-induced degranulation, suggesting that arrestins regulate exocytosis in neutrophils by activating Src family tyrosine kinases. Similarly, the translocation of vesicles containing the glucose transporter, Glut4, to the plasma membrane in response to endothelin type A receptor activation is dependent on assembly of an arrestin2 complex with c-Yes (Imamura et al. 2001).

4 Conclusions

Whereas GPCR signals transmitted through heterotrimeric G protein-arrestin second messenger pathways, with their rapid onset and termination kinetics, are ideal for minute-to-minute regulation of intermediary metabolism, signals transmitted through arrestin-based signalsomes provide longer-term control of pathways regulating such processes as cell proliferation, survival, and chemotaxis. Because of its dual desensitizing and signaling functions, arrestin binding marks the transition of a GPCR between two temporally, spatially, and functionally discrete signaling states; a short-lived G protein-coupled state on the plasma membrane and a more durable arrestin-coupled state that continues to signal as the "desensitized" GPCR transits the endosomal compartment. It is now recognized that regulation of ERK1/2 and regulation of Src family kinases are but two of the several physiologically important arrestin-dependent signaling events and that others, e.g., regulation of cell survival and apoptosis (*see* chapters "Targeting Individual GPCRs with Redesigned Non-visual Arrestins" and "Self-Association of Arrestin Family Members") and cytoskeletal rearrangement and migration (*see* chapters "β-Arrestins and G Protein-Coupled Receptor Trafficking" and "Self-Association of Arrestin Family Members"), may have equal or greater impact on the cellular response to GPCR activation. Given this growing recognition, it is not surprising that pharmacologic manipulation of arrestin signaling, whether through arrestin- or G protein-selective "biased" agonists (*see* chapter "Quantifying Biased β-Arrestin Signaling") or gene therapy with functionally specialized arrestin mutants (*see* Chapters "Therapeutic Potential of Small Molecules and Engineered Proteins," "Enhanced Phosphorylation-Independent Arrestins and Gene Therapy," "Targeting Individual GPCRs with

Redesigned Non-visual Arrestins," "Arrestin-Dependent Activation of JNK Family Kinases," and "Arrestins in Apoptosis"), has gained attention. The expectation for the future is that the ability to manipulate the heterotrimeric G protein and arrestin signaling networks independently will offer novel therapies for such diverse conditions as heart disease, asthma, inflammation, osteoporosis, and cancer.

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Arrestin-Dependent Activation of JNK Family Kinases

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Contents

Abstract The activity of all mitogen-activated protein kinases (MAPKs) is stimulated via phosphorylation by upstream MAPK kinases (MAPKK), which are in their turn activated via phosphorylation by MAPKK kinases (MAPKKKs). The cells ensure the specificity of signaling in these cascades by employing a variety of scaffolding proteins that bind matching MAPKKKs, MAPKKs, and MAPKs. All four vertebrate arrestin subtypes bind JNK3, but only arrestin-3 serves as a scaffold, promoting JNK3 activation in intact cells. Arrestin-3-mediated JNK3 activation does not depend on arrestin-3 interaction with G protein-coupled receptors (GPCRs), as demonstrated by the ability of some arrestin mutants that cannot bind receptors to activate JNK3, whereas certain mutants with enhanced GPCR binding fail to promote JNK3 activation. Recent findings suggest that arrestin-3 directly binds both MAPKKs necessary for JNK activation and facilitates JNK3

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phosphorylation at both Thr (by MKK4) and Tyr (by MKK7). JNK3 is expressed in a limited set of cell types, whereas JNK1 and JNK2 isoforms are as ubiquitous as arrestin-3. Recent study showed that arrestin-3 facilitates the activation of JNK1 and JNK2, scaffolding MKK4/7-JNK1/2/3 signaling complexes. In all cases, arrestin-3 acts by bringing the kinases together: JNK phosphorylation shows biphasic dependence on arrestin-3, being enhanced at lower and suppressed at supraoptimal concentrations. Thus, arrestin-3 regulates the activity of multiple JNK isoforms, suggesting that it might play a role in survival and apoptosis of all cell types.

Keywords Arrestin • JNK • Scaffold • Cell signaling • Protein phosphorylation • Apoptosis

1 The Discovery of the Role of Arrestins in JNK Activation

Arrestins are best known for their ability to specifically bind active phosphorylated forms of their cognate G protein-coupled receptors (GPCRs) (Carman and Benovic 1998; Gurevich and Gurevich 2006b; Gurevich et al. 2011). Arrestin-3 binding to MAP kinase c-Jun N-terminal kinase 3 (JNK3) and its upstream MAP kinase kinase kinase (MAP3K) apoptosis signal-regulating kinase 1 (ASK1) was described in 2000 (McDonald et al. 2000), within a few years of the discovery of the first non-receptor-binding partner of arrestin, clathrin (Goodman et al. 1996).

Using co-immunoprecipitation (co-IP), the authors of the original study (McDonald et al. 2000) demonstrated that one of the nonvisual subtypes, β-arrestin2 (systematic name arrestin-3[1]), is found in a complex with JNK3, mitogen-activated protein kinase kinase (MAP2K) 4 (MKK4), and ASK1, which together constitute one of the typical three-kinase MAPK activation modules, ASK1–MKK4–JNK3. The results of co-IP from differentially transfected cells suggested that arrestin-3 bound ASK1 and JNK3, whereas MKK4 was brought to the complex via interactions with the other two kinases. The authors also found that the stimulation of the angiotensin II type 1A receptor increased JNK3 phosphorylation in transfected cells and triggered the colocalization of arrestin-3 and active phospho-JNK3 to cytoplasmic vesicles (McDonald et al. 2000). This leads to the hypothesis that arrestins function as receptor-regulated MAPK scaffolds, promoting JNK3 phosphorylation and localizing active JNK3 to ligand-activated GPCRs. However, follow-up study from the same group showed that receptor is not

[1] Different systems of arrestin names are used in the field and in this book. We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons, its gene is called "*arrestin-3*" in the HUGO database).

obligatory for this function of arrestin-3: it effectively facilitated JNK3 phosphorylation in cells overexpressing ASK1 in the absence of receptor activation (Miller et al. 2001).

Both of these studies suggested that arrestin-3 is the only nonvisual subtype that binds JNK3 and its upstream kinases, so the fact that arrestin-2 does not promote JNK3 activation was explained by the lack of binding (McDonald et al. 2000; Miller et al. 2001). However, arrestin-2 and arrestin-3 are highly homologous (Gurevich and Gurevich 2006a), and both appear to bind numerous GPCRs (Gurevich et al. 1995; Barak et al. 1997), clathrin (Goodman et al. 1996), clathrin adaptor AP2 (Laporte et al. 1999), and protein kinase c-Src (Luttrell et al. 1999) comparably. Therefore, the issue of the binding of different arrestin subtypes to JNK3 and upstream kinases was further investigated (Song et al. 2006, 2007, 2009).

2 Which Kinases Bind Which Arrestin Subtypes?

Earlier observations showed that arrestin-3 redistributes JNK3 from the nucleus to the cytoplasm (Scott et al. 2002) and that this phenomenon requires functional nuclear export signal (NES) in the C terminus of arrestin-3 (Wang et al. 2003). Based on these findings, it was demonstrated that a single amino acid substitution in the C terminus of arrestin-2 that creates functional NES results in similar removal of JNK3 from the nucleus in the presence of NES-positive arrestin-2, demonstrating that arrestin-2 actually binds JNK3 (Song et al. 2006). Moreover, visual arrestin-1 (Song et al. 2006) and arrestin-4 (Song et al. 2007) were found to redistribute JNK3 in the cell as efficiently as nonvisual subtypes, contradicting the idea that JNK3 binding is a unique feature of arrestin-3 (McDonald et al. 2000) and demonstrating that all vertebrate arrestins bind JNK3. Obviously, JNK3 activation requires simultaneous recruitment of upstream kinases, so inability of other subtypes to bind ASK1 and MKK4/7 could explain why only arrestin-3 promotes JNK3 activation. However, this explanation also did not survive experimental testing: both arrestin-2 (Song et al. 2009; Seo et al. 2011) and arrestin-1 (Gurevich et al. 2011) were shown to bind ASK1 and MKK4, similar to arrestin-3. Reconstitution of MKK4-JNK3α2 signaling module with arrestin-2 and arrestin-3 from purified proteins suggested that the difference might be in affinity: arrestin-3 showed tighter binding of both JNK3α2 and MKK4 than arrestin-2 (Zhan et al. 2011b). However, a subsequent study described arrestin-3 mutant with higher affinity for JNK3α2 than WT, which also comparably bound MKK4 and ASK1, but failed to promote JNK3α2 phosphorylation in cells (Breitman et al. 2012). Thus, while difference in affinity might play a role in differential effects of scaffolding of MAP kinase cascades by the two nonvisual arrestins, it is certainly not the only factor. Extensive structure–function studies of the ability of arrestin-3 to facilitate JNK3α2 phosphorylation in intact cells (Seo et al. 2011; Breitman et al. 2012) suggest that an arrestin can bind all required kinases, but hold them in "wrong" orientation, which is not conducive to signaling.

3 Does Arrestin Conformation Affect Its Interactions with JNK3 and Upstream Kinases?

Nonvisual arrestins interact with a fairly diverse group of non-receptor-binding partners, including proteins involved in receptor trafficking, protein kinases, E3 ubiquitin ligases and deubiquitinating enzymes, small G proteins, etc. (see chapters "β-arrestins and G Protein-Coupled Receptor Trafficking," "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications," "Arrestin-Dependent Activation of ERK and Src Family Kinases," "Arrestin-Dependent Activation of JNK Family Kinases," "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences," "Arrestin-Dependent Localization of Phosphodiesterases," "Arrestins in Apoptosis," "Molecular Mechanisms Underlying Beta-Arrestin-Dependent Chemotaxis and Actin-Cytoskeletal Reorganization," "Arrestins in Host-Pathogen Interactions," and "Arrestin Regulation of Small GTPases"). Ever-expanding repertoire of arrestin-binding proteins has been identified at a fast pace since the discovery of the first non-receptor partner, clathrin, in 1996 (Goodman et al. 1996). In a recent proteomic analysis, 71 proteins were reported to bind arrestin-2, 162 proteins bound arrestin-3, and 102 proteins interacted with both nonvisual arrestins (Xiao et al. 2007). Interestingly, some proteins prefer receptor-bound arrestin conformation and others preferentially bind arrestins in their basal state, whereas some do not have an obvious preference (Luttrell et al. 2001; Song et al. 2006, 2009; Ahmed et al. 2011; Coffa et al. 2011b). Although arrestin often functions as scaffold or adaptor by tethering multiple components together, a single receptor-bound arrestin is not big enough to accommodate more than 4–6 partners simultaneously (Gurevich and Gurevich 2006b). Thus, many of the binding partners have to compete with each other for arrestin. How arrestin makes the "decision" to interact with the "right" set of partners remains a very interesting and challenging question. Arrestins undergo significant conformational changes upon binding different partners (Schleicher et al. 1989; Hanson et al. 2006a; Kim et al. 2012; Zhuang et al. 2013). Arrestins are known to exist in at least three distinct conformations: free, revealed by most crystal structures (Granzin et al. 1998; Hirsch et al. 1999; Han et al. 2001; Milano et al. 2002; Sutton et al. 2005; Zhan et al. 2011a), receptor-bound (Palczewski et al. 1991; Kim et al. 2012; Zhuang et al. 2013), and microtubule-bound (Hanson et al. 2006a, 2007). Upon binding to the receptor, arrestin undergoes a global conformational change (Schleicher et al. 1989; Gurevich and Benovic 1993; Hanson et al. 2006b; Kim et al. 2012; Zhuang et al. 2013). This rearrangement alters the set of exposed elements, thereby affecting the association of arrestins with their non-receptor-binding partners (Ahmed et al. 2011). Binding-induced conformational changes in arrestin could play decisive role in arrestin-mediated assembly of distinct signaling complexes in various physiological conditions (Ahmed et al. 2011). Two recent structures, one of truncated form of arrestin-2 [first described as "pre-activated" mutant in Celver et al. (2002)]

associated with multi-phosphorylated C-terminus of vasopressin V2 receptor (Shukla et al. 2013) and the other of short arrestin-1 splice variant p44 also lacking the C-tail (Kim et al. 2013), are remarkably similar. Importantly, arrestin in both is not in complex with the receptor, for which it has sub-nanomolar to nanomolar affinity (Gurevich et al. 1993, 1995; Bayburt et al. 2011). Instead, it is either associated with phosphopeptide (Shukla et al. 2013), which has orders of magnitude lower affinity for arrestin, or not associated with any part of the receptor (Kim et al. 2013). It has been previously shown that the more "pre-activating" mutations are introduced into arrestin, the less stable the protein becomes (Schleicher et al. 1989; Hirsch et al. 1999; Sutton et al. 2005), suggesting that it is highly unlikely that fully activated arrestin conformation can be stable without bound receptor. Therefore, these structures likely do not represent the active receptor-bound conformation, but an intermediate step on the way to it. Nonetheless, these structures are very informative. Relative to the basal state, both feature a significant movement of the "139-loop," previously shown to change position in response to receptor binding (Schleicher et al. 1989; Hirsch et al. 1999; Kim et al. 2012) and act as a "brake" reducing arrestin binding to non-preferred forms of the receptor (Vishnivetskiy et al. 2013), and the twisting of the two domains relative to each other by 20–21° (Kim et al. 2013; Shukla et al. 2013). Previous studies showed that an extended inter-domain hinge is necessary for receptor binding of arrestin-1 (Vishnivetskiy et al. 2002), as well as nonvisual arrestin-2 and arrestin-3 (Hanson et al. 2007). However, proposed on the basis of these data, clam-like domain movement (Gurevich and Gurevich 2004) was not detected by intramolecular distance measurements using pulse EPR technique DEER (Kim et al. 2012). The twisting of the two domains revealed by these structures (Kim et al. 2013; Shukla et al. 2013) represents an alternative movement that explains the requirement of a certain length of the hinge, which was suggested by reported deleterious effect of hinge deletions on receptor binding (Vishnivetskiy et al. 2002; Hanson et al. 2007).

Although arrestin was originally shown to act as a receptor-regulated scaffold for JNK3 activation (McDonald et al. 2000), the follow-up studies indicated that receptor stimulation is not obligatory for this arrestin function (Miller et al. 2001). JNK3 interactions with different arrestin conformations: basal, constitutively "pre-activated" form [3A mutant with forcibly detached C terminus (Gurevich 1998; Carter et al. 2005)]; and the mutant with the deletion of seven residues in the inter-domain hinge that is impaired in receptor binding (Δ7) (Vishnivetskiy et al. 2002; Hanson et al. 2007) were evaluated in a nuclear exclusion assay, based on the ability of arrestins with functional NES to remove their binding partners from the nucleus to the cytoplasm (Song et al. 2006, 2007). WT arrestins, as well as "pre-activated" 3A and non-receptor-binding Δ7 mutants, effectively relocated JNK3 (Song et al. 2006), indicating that JNK3 binding does not depend on arrestin conformation. Furthermore, the Δ7 mutant robustly promoted JNK3 activation, at least as well as WT (Song et al. 2009), whereas another mutant termed KNC, in which receptor interaction was precluded by the elimination of key GPCR-binding residues, demonstrated stronger binding to JNK3 than WT arrestin-3 (Breitman et al. 2012). Recently, we demonstrated direct JNK3 interaction with arrestins in

the basal conformation using purified proteins in vitro (Zhan et al. 2011b, 2013). Importantly, in this strictly controlled system reconstructed from pure proteins, free arrestin-3 in the absence of any receptor functioned as a scaffold facilitating JNK3 phosphorylation by both MKK4 (Zhan et al. 2011b) and MKK7 (Zhan et al. 2013). However, these studies did not test the effect of arrestin binding to the receptor on its interactions with JNK3 or upstream kinases. To evaluate the effect of receptor on arrestin-JNK3 interaction, careful biochemical experiments with purified proteins need to be performed to measure the binding affinities of JNK3, MKK4, MKK7, and ASK1 for arrestin-3 in its basal conformation and bound to the receptor, as well as the efficiency of arrestin-3-mediated scaffolding this signaling cascade in the presence and absence of GPCRs.

Arrestins were reported to facilitate the activation of three main subfamilies of MAP kinases: JNK (McDonald et al. 2000), ERK (Luttrell et al. 2001), and p38 (Bruchas et al. 2006). In each cascade, in order to promote signaling, arrestins need to assemble appropriate combinations of MAPK, MAPKK, and MAPKKK. It is not clear how simultaneous association of mismatched kinases that would create unproductive complexes is prevented. One possibility, based on previous observations, is that the binding of one kinase to arrestin can significantly alter the recruitment of another. For example, MKK4, MAPKK in JNK3 activation cascade, demonstrates weaker binding to arrestin than ASK1 and JNK3 (McDonald et al. 2000; Song et al. 2009; Breitman et al. 2012). The binding of ASK1, JNK3, or both dramatically enhances MKK4 association with arrestin (McDonald et al. 2000; Song et al. 2009). Our recent studies showed that JNK3 binding differentially modulates the recruitments of the two upstream MAPKKs, MKK4 and MKK7, to arrestin-3, enhancing the binding of MKK4 while reducing that of MKK7 (Zhan et al. 2013). Although these results were obtained with kinases from the same module, the data suggest that interdependence of the binding of MAP kinases likely contributes to the assembly of signaling complexes containing matching kinases.

4 The Arrangement of ASK1, MKK4, and JNK3 on Arrestin

Arrestin-mediated JNK3 activation requires simultaneous recruitment of JNK3 and its upstream kinases. The original work (McDonald et al. 2000) proposed a model where both ASK1, the MAPKKK in JNK3 cascade, and JNK3 directly associate with arrestin-3, whereas MKK4 is recruited via ASK1 and/or JNK3 without directly interacting with arrestin-3. This model was mainly based on the observation that the interaction between MKK4 and arrestin-3 could not be detected by co-IP unless MKK4 was co-expressed with ASK1, JNK3, or both. However, direct interaction between MKK4 and arrestin-3 was demonstrated in follow-up studies by several different assays including co-IP (Song et al. 2009), direct pull-down using purified

Fig. 1 The arrangement of the three kinases on arrestin scaffold. MAPK activation modules consist of MAPKKK, MAPKK, and MAPK. The model shown is based on several lines of evidence. First, each of the three kinases in ASK1–MKK4–JNK3 and c-Raf1–MEK1–ERK2 signaling modules was shown to bind both arrestin domains (Song et al. 2009). Second, many purified kinases, such as JNK3 and MKK4 (Zhan et al. 2011b), MKK7 (Zhan et al. 2013), JNK1 and JNK2 (Kook et al. 2013), as well as MEK1 and ERK2 (Coffa et al. 2011a), were shown to bind arrestins directly. Receptor binding-deficient arrestin-3 mutant with 7-residue deletion in the inter-domain hinge facilitates ASK1-dependent JNK3 activation at least as efficiently as WT arrestin-3 (Song et al. 2009; Breitman et al. 2012), whereas arrestin-3-3A mutant with enhanced receptor binding (Celver et al. 2002) does not promote JNK3 activation (Breitman et al. 2012). Therefore, the model shows that not only receptor-bound but free arrestins can also scaffold MAP kinase cascades. Arr, arrestin scaffold; ASK, ASK1 (or any MAPKKK); MKK, MKK4/7 (or any MAPKK); J, JNK1/2/3 (or any MAPK)

proteins (Zhan et al. 2011b, 2013; Kook et al. 2013), and MKK activity assay in reconstituted arrestin-3–MKK4–JNK3 (Zhan et al. 2011b), arrestin-3–MKK7–JNK3 (Zhan et al. 2013), as well as arrestin-3–MKK4/7–JNK1/2 (Kook et al. 2013) systems. Moreover, separated arrestin-3N- and C-domains were shown to bind each kinase in the ASK1–MKK4–JNK3 module (Song et al. 2009). Interestingly, the kinases of the ERK1/2 module (c-Raf1, MEK1, and ERK2) also bind equally well to both domains of arrestin-2 and arrestin-3 (Song et al. 2009). This appears to be a universal mode of assembly of the three kinases in MAPK cascades on arrestin scaffolds. Therefore, a different model of the MAPK cascade organized by arrestin has been proposed, which is based on the identification of multiple arrestin-binding elements in MAP kinases and localization of binding elements for each kinase on both N- and C-domains of nonvisual arrestins (Fig. 1). In this model, arrestin binds all three kinases directly, with each kinase interacting with both domains of arrestin. In fact, since the two arrestin domains apparently move relative to each other upon its binding to GPCRs (Schleicher et al. 1989; Gurevich and Gurevich 2004) and microtubules (Hanson et al. 2006a), the most straightforward mechanism that would make the binding of any protein sensitive to arrestin conformation is the engagement of elements in

both arrestin domains. Although JNK3 is a rare example of a binding partner that does not show a clear preference for a particular arrestin conformation (Song et al. 2006, 2007), it appears to follow this general rule.

5 Binding and Activation Are Two Distinct Phenomena

Earlier observations showed that arrestin-2 binds all three components in the ASK1–MKK4–JNK3 cascade (Song et al. 2009; Seo et al. 2011). However, in contrast to highly homologous arrestin-3 (Hanson et al. 2007; Ahmed et al. 2011), arrestin-2 and the two visual arrestins fail to facilitate the activation of JNK3 (Song et al. 2009). Thus, the ability to bind these kinase components simultaneously does not ensure the facilitation of JNK3 activation. The two nonvisual arrestins are highly homologous: bovine arrestin-2 and arrestin-3 have 78 % sequence identity and 88 % similarity (Sterne-Marr et al. 1993) and their crystal structures are also remarkably similar, particularly in the core arrestin fold (Han et al. 2001; Zhan et al. 2011a). Due to high structural similarity, arrestin chimeras usually remain fully functional (Gurevich et al. 1993, 1995; Vishnivetskiy et al. 2004; Hanson et al. 2006a; Ahmed et al. 2011). Several arrestin-2/3 chimeras and mutants constructed by swapping the elements between the two nonvisual arrestins were used to explore the structural basis of the ability of arrestin-3, but not arrestin-2, to facilitate JNK3 activation (Seo et al. 2011). Both domains of arrestin were found to contribute to JNK3 activation, with the C-domain being more important than the N-domain (Seo et al. 2011). In addition, it was shown that several residues on the non-receptor-binding side of arrestin-3 are critical for JNK3 activation: Val-343 is the key contributor to this function, whereas Leu-278, Ser-280, His-350, Asp-351, His-352, and Ile-353 play supporting roles (Seo et al. 2011). However, in contrast to many of arrestin-3 mutants containing arrestin-2 residues that have lost the ability to facilitate JNK3 phosphorylation, the efforts to build this function into arrestin-2 by replacing these critical residues with the corresponding ones from arrestin-3 were not particularly successful (Seo et al. 2011). Interestingly, virtually all arrestin-3 mutants deficient in their ability to promote JNK3 phosphorylation were shown to bind ASK1, MKK4, and JNK3 normally (Seo et al. 2011).

The observations that arrestin binding of kinases of JNK3 signaling cascade does not necessarily translate into the ability to facilitate JNK3 activation suggest that to promote signaling arrestin has to assemble the kinases in optimal relative orientation. Several arrestin-3 mutants have been generated which bind ASK1, MKK4, and JNK3 at least as well as WT arrestin-3 but fail to facilitate JNK3 activation (Seo et al. 2011; Breitman et al. 2012). Arguably, the most interesting and potentially useful of these is arrestin-3-KNC mutant, which binds JNK3 even better than WT and interacts with the two upstream kinases normally, but does not facilitate JNK3 activation in cells (Breitman et al. 2012). Because KNC mutant binds ASK1, MKK4, and JNK3 very effectively, it competes with WT arrestin-3 and other JNK scaffold proteins, thereby acting as a “silent scaffold,” suppressing JNK3 activation in the cell (Breitman et al. 2012).

To improve our understanding of the relationship between binding and scaffolding functions of arrestins, the interactions of arrestin-3 with each kinase in the cascade need to be characterized quantitatively. The relative orientation of the three kinases within the complex with arrestin-3 might be critical for JNK3 activation, but the differences in binding affinities can play an important role, as well. Most of the studies reporting the binding of arrestin to the kinases in JNK3 cascade used co-immunoprecipitation or cell-based BRET assays (McDonald et al. 2000; Song et al. 2009; Seo et al. 2011; Breitman et al. 2012). Considering that in intact cells arrestins can interact with wide variety of proteins (Xiao et al. 2007), it is hard to derive actual binding affinities from these largely qualitative observations or even ascertain that the interactions between arrestins and their partners are direct. To compare the interactions of WT arrestins and their mutants with each kinase, binding affinities should be measured directly using purified proteins. For example, relatively weak interactions of arrestins with MKK4 are hard to measure, and this binding is regulated significantly by the presence or absence of other kinases (Zhan et al. 2013). The MKK4 binding to the two nonvisual arrestins was believed to be comparable until the direct pull-down assay with purified proteins suggested otherwise: arrestin-3 binds MKK4 with higher affinity than arrestin-2 (Zhan et al. 2011b). These differences in binding affinity are consistent with the functional assay with reconstructed arrestin–MKK4–JNK3 modules, in which optimal concentration of arrestin-2 for maximal JNK3 activation was much higher than that of arrestin-3 (Zhan et al. 2011b). The optimal concentration of arrestins, and other scaffolds, is highly dependent on the binding affinities of the components (Burack and Shaw 2000).

Bringing all components into close proximity and holding them in optimal orientation are the two basic functions of scaffolding proteins that facilitate signal transduction (Dhanasekaran et al. 2007; Good et al. 2011). Tethering increases the effective local concentration of enzymes and their substrates, prevents the competition of other molecules, and keeps the specificity in signal transduction. Scaffold proteins can also direct the signal transduction through properly orienting target proteins with upstream enzymes, accelerating reactions manifold (Schlosshauer and Baker 2004). The evidence suggests that binding affinities along with the ability to ensure proper orientation of the kinases involved contribute to the subtype-specific facilitation of JNK3 activation by arrestin-3.

6 Reconstruction of Arrestin–MAP2K–MAPK Modules from Pure Proteins: Arrestins Are True Scaffolds

Although cell-based systems have been valuable tools to probe the roles of scaffold proteins in MAP kinase signaling, they have their limits. First, the number of exogenous components introduced into the cell is limited. As more cDNAs are introduced to study the signaling complex, co-transfection by all becomes less

certain. Second, it is hard to control the expression level of each component, which changes upon co-expression of others, so that the amount of cDNA used needs to be adjusted for each combination. Besides, some proteins are less stable than others, which limits their expression levels, sometimes to the point of making their effects undetectable (Breitman et al. 2012). Third, it is virtually impossible to avoid the interference from unknown factors in the cellular milieu. Strictly controlled systems reconstructed from purified proteins avoid many inherent problems of cell-based assays, and often provide more direct and quantitative answers, helping to elucidate the underlying molecular mechanisms.

The reconstruction of arrestin-MKK4-JNK3 module from purified proteins has been reported recently, and the effects of arrestins on JNK3 phosphorylation by MKK4 have been carefully examined (Zhan et al. 2011b). JNK3α2 phosphorylation was shown to be a biphasic function of the concentration of both nonvisual arrestins. At lower arrestin concentrations, the JNK3α2 phosphorylation was enhanced, but it was inhibited by higher concentrations (Zhan et al. 2011b). Previous mathematical modeling suggested that the biphasic dependence of signaling efficiency on the concentration of a scaffold is observed when scaffold protein binds each component directly (Levchenko et al. 2000, 2004). Experiments with pure proteins proved that arrestin binds both MKK4 and JNK3 simultaneously, forming a ternary complex (Zhan et al. 2011b). In the reconstructed system (Fig. 2), JNK3 and MKK4 can exist in three states: associated in solution without scaffolds (JM), bound to scaffold protein not associated with the other kinase to form an incomplete complex (AM and AJ), and bound to scaffold simultaneously with the other kinase in an active signaling complex (JAM). Comparing to JM formed when JNK3 and MKK4 encounter each other by diffusion, the complete signaling complex JAM is a high output pathway, whereas the formation of incomplete complexes AM and AJ reduces the probability of the interaction of freely diffusing MKK4 and JNK3. An increase in scaffold concentration in the lower range enhances the formation of JAM complex, thereby facilitating the activation of the downstream kinase. In contrast, a further increase in scaffold concentration increases the probability of downstream and upstream kinase associating with the scaffold protein alone, forming incomplete inactive complexes (AM and AJ in Fig. 2). Therefore, the activation of MAPK by its upstream kinase can be inhibited by high concentrations of scaffolds. This has been demonstrated experimentally for kinase suppressor of Ras 1 (KSR1)-dependent ERK activation (Kortum and Lewis 2004), as well as JNK1/2/3 activation assisted by arrestin-3 (Zhan et al. 2011b, 2013; Kook et al. 2013). The optimal scaffold concentration for maximal JNK3 activation is highly dependent on several parameters. One is the binding affinity of the two kinases for arrestin and possible cooperativity between the binding of JNK3 and MKK4 to the scaffold. Stronger binding (lower K_D) results in lower optimal scaffold concentration (Zhan et al. 2013). Therefore, higher optimal concentration of arrestin-2 than arrestin-3 observed in vitro was consistent with the direct binding assay, where arrestin-3 demonstrated stronger binding for MKK4 and JNK3 than arrestin-2 (Zhan et al. 2011b).

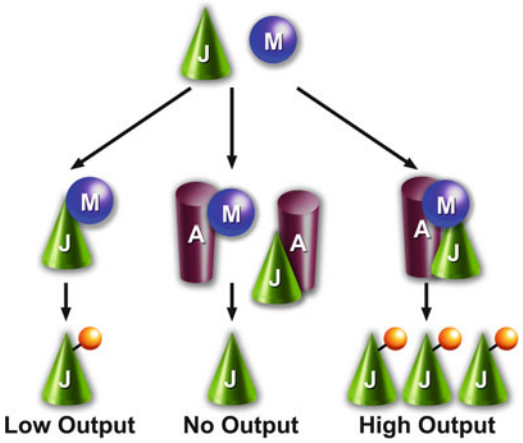

Fig. 2 Scaffold concentration matters. Signaling can occur in the absence of scaffolds (the formation of JM complex; low output) and optimum scaffold concentrations promote the formation of complete complexes (JAM), thereby facilitating signaling (high output), whereas supraoptimal levels of scaffold make the formation of incomplete complexes (JA and AM) more likely, suppressing signaling in the cascade (no output). Biphasic effect of scaffold concentration on signaling was predicted by mathematical modeling (Levchenko et al. 2000, 2004) and experimentally demonstrated in case of arrestin-3 scaffolding of signaling modules that activate JNK family kinases (Zhan et al. 2011b, 2013; Kook et al. 2013). A, arrestin-3; M, MKK4/7; J, JNK1/2/3

The reconstruction of signaling system from pure proteins also proved the existence of another relevant module scaffolded by arrestin-3: arrestin-3–MKK7–JNK3. JNK family kinases are activated by concomitant phosphorylation of a threonine and a tyrosine residue within a conserved Thr–Pro–Tyr (TPY) motif in the activation loop of the kinase domain. Two upstream MAP kinase kinases, MKK4 and MKK7, preferentially phosphorylate distinct JNK activation sites: MKK4 phosphorylates tyrosine, whereas MKK7 phosphorylates threonine (Lawler et al. 1998). Arrestin-3-dependent increase in the phosphorylation of Thr on JNK3α2 was demonstrated both in vitro and in intact cells (Zhan et al. 2013), which suggested that arrestin-3 can recruit MKK7 to activate JNK3 as well. Direct interaction between arresitn-3 and MKK7 in the absence of JNK3 has been confirmed by pull-down assay (Zhan et al. 2013). The binding to arrestin-3 of the active (phosphorylated) MKK4 and MKK7 was also evaluated in the reconstructed system. Both active MKKs bind arrestin-3 at the level comparable to that of inactive forms of these two kinases (Zhan et al. 2013). This is consistent with the model that these MKKs bind arrsetin-3 along with their upstream activator ASK1, which then phosphorylates them, whereupon they can phosphorylate their downstream substrates, JNKs. Unexpectedly, we found that the binding of downstream kinase JNK3 differentially affects the recruitment of these two MKKs, enhancing the binding of MKK4, while decreasing the binding of MKK7 (Zhan et al. 2013). As could be expected (Fig. 2), the activation of JNK3 by MKK7 demonstrated biphasic dependence on arrestin-3 concentration (Zhan et al. 2013), similar to that observed with MKK4 (Zhan et al. 2011b). However,

arrestin-3 concentration optimal for JNK3 phosphorylation by MKK7 was eight- to tenfold higher than in case of MKK4 (Zhan et al. 2013). This finding is in excellent agreement with the negative cooperativity revealed by direct binding assay (Zhan et al. 2013). Mathematical models predict that positive binding cooperativity between the two components lowers the optimal scaffold concentration, whereas negative cooperativity increases the optimal scaffold concentration (Bray and Lay 1997; Ferrell 2000; Levchenko et al. 2000, 2004).

Thus, experiments with pure proteins yielded information that remained unattainable otherwise. Reconstruction of complete MAPKKK–MAPKK–MAPK modules with pure proteins in the presence or absence of arrestins must be performed next. The situation involving three kinases that sequentially phosphorylate and activate each other is more complex. Biochemical assays under strictly controlled conditions are powerful tools for elucidating the mechanisms of arrestin-mediated scaffolding of MAP kinase cascades. So far, these studies were limited to MAPKK–MAPK modules (Zhan et al. 2011b, 2013; Kook et al. 2013). The addition of MAPKKK and other regulators, such as GPCRs, will provide novel valuable information.

7 Suppression of JNK3 Activation by Arrestin-3-Based Silent Scaffold

JNK signaling is involved in the normal physiological processes of cell proliferation, apoptosis, differentiation, and cell migration (Davis 2000; Karin and Gallagher 2005). JNK3, a brain-specific isoform of JNKs, has been implicated in neurodegenerative diseases, such as Parkinson's (Hunot et al. 2004), Alzheimer's (Yoon et al. 2013), and Huntington's (Morfini et al. 2009). These studies suggest that inhibition of JNK3 activity could be a promising therapeutic intervention for neurodegenerative diseases.

JNK3, like other MAP kinases, is activated by MAPKKs (concerted action of MKK4 or MKK7 is required in this case) (Lawler et al. 1998; Tournier et al. 2001), which in turn are activated by MAPKKKs (Davis 2000). To confer spatial and temporal regulation of the JNK signaling by multiple stimuli, cells have developed a class of proteins that function as specialized scaffolds. Protein scaffolds assemble signaling modules by binding multiple components of the MAPK cascade, bringing them into close proximity, thereby facilitating efficient propagation of the signal (Brown and Sacks 2009). Arrestin-3 was reported to function as scaffold protein for JNK3 signaling, promoting JNK3 phosphorylation by binding ASK1, MKK4, MKK7, and JNK3 (McDonald et al. 2000; Miller et al. 2001; Song et al. 2009; Seo et al. 2011; Zhan et al. 2011b, 2013). Several arrestin-3 mutants bind ASK1, MKK4, and JNK3 at least as well as WT arrestin-3, but do not facilitate JNK3 phosphorylation in cells (Seo et al. 2011). These data suggest that one can engineer

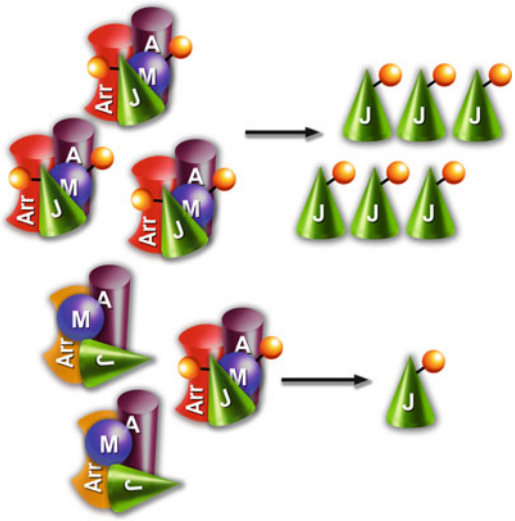

Fig. 3 Silent scaffolds can suppress MAPK signaling. *Upper panel*. By assembling multi-kinase complexes, scaffolds can greatly enhance signaling in MAPK pathways (Brown and Sacks 2009). *Lower panel*. Scaffolding proteins with targeted mutations that bind all kinases but do not facilitate their activation by sequential phosphorylation recruit the kinases away from endogenous productive scaffolds, thereby acting as dominant-negative "silent scaffolds" that suppress the signaling in the cascade (Breitman et al. 2012). The simplest conceivable mechanism of this effect is that silent scaffolds bind kinases but hold them in "wrong" configuration that is not conducive to phosphorylation, which is reflected by different arrangement of the kinases on the silent scaffold shown. Arr *red*, WT arrestin (or any productive scaffold); Arr *yellow*, mutant arrestins (or any reengineered scaffold that binds kinases but does not promote the signaling); A, ASK1 (or any MAPKKK); M, MKK4/7 (or any MAPKK); J, JNK1/2/3 (or any MAPK)

an arrestin-3 mutant that suppresses the signaling in this cascade simply by recruiting the kinases into unproductive complexes, thereby keeping them away from productive scaffolds (Fig. 3).

This idea was recently tested and found to be correct (Breitman et al. 2012). Arrestin-3-KNC mutant, where 12 known receptor-binding residues were replaced with alanines (Vishnivetskiy et al. 2011; Gimenez et al. 2012), binds upstream kinases ASK1 and MKK4 normally and demonstrates stronger binding to JNK3 than WT arrestin-3 in cells, as shown by both arrestin-3-JNK3 BRET and co-immunoprecipitation, but does not have the ability to promote JNK3 activation (Breitman et al. 2012). As expected, arrestin-3-KNC mutant acts as a dominant-negative silent scaffold (Fig. 3): its increasing expression progressively decreases JNK3 phosphorylation in the cell induced by WT arrestin-3, apparently via sequestration of all three kinases into unproductive complexes (Breitman et al. 2012). Many experimental and modeling studies suggested that selective modulation of the interaction between scaffolds and individual targets might enable specific regulation of MAPK activity, directing the cellular response towards (or away from) a

particular function, without attenuating global MAPK activity (Brown and Sacks 2009). However, arrestin-3-KNC is the first molecular tool that was developed on the basis of this concept, specifically for the suppression of JNK3 signaling in living cells.

Spatial and temporal changes in MAPK signaling affect cellular response to a specific stimulus and are very important for biological specificity of MAPKs. Many scaffold proteins appear to have a fundamental role in the spatial regulation of MAPK signaling (Morishima-Kawashima and Kosik 1996; Li et al. 2005; Brown and Sacks 2009). Most of activated JNK family kinases tend to translocate into the nucleus and phosphorylate nuclear substrates, such as c-Jun, anti-activating transcription factor (ATF-1), JunB, and JunD, thereby inducing JNK-dependent gene expression (Bogoyevitch and Kobe 2006). Interestingly, JNK3 activated by arrestin-3 remains in the cytosol (McDonald et al. 2000; Breitman et al. 2012), where it likely phosphorylates other cellular proteins, possibly those implicated in apoptotic cell death, such as Bcl2, p53, etc. (Fuchs et al. 1998; Yamamoto et al. 1999; Buschmann et al. 2001; Deng et al. 2001; Bogoyevitch and Kobe 2006). In cells expressing arrestin-3-KNC or arrestin3-3A mutants which do not facilitate JNK3 activation and likely inhibit its activity in the cytosol, most of phospho-JNK (likely activated via arrestin-independent mechanisms) was detected in nucleus (Breitman et al. 2012). Thus, arrestin-3-KNC and other cytoplasmic silent scaffolds appear to be suitable tools to regulate cellular JNK3 activity in compartment-specific manner, whereas similar dominant-negative scaffolds with nuclear localization can regulate MAPK signaling specifically in that compartment. Arrestin-3-KNC is not a perfect tool for targeted manipulation of JNK3-specific signaling, because it simultaneously decreases the activation of both JNK3 and ERK1/2 (Breitman et al. 2012). Nonetheless, the development and functional validation of the arrestin-3-based silent scaffold is an important proof-of-concept experiment. The construction of molecular tools of this type creates new methods for precisely targeted spatial and temporal regulation of MAPK signaling. Further experiments are necessary to test whether arrestin-3-KNC similarly reduces the activation of JNK1/2 in the cell and whether the suppression of JNK signaling by this mutant translates into increased cell survival in culture and in vivo.

8 Arrestin-Dependent Activation of JNK1/2 Isoforms

The interaction of arrestin-3 and JNK3 was originally detected in yeast two-hybrid screen and confirmed using co-immunoprecipitation from cultured cells (McDonald et al. 2000). Subsequently, the ability of arrestin-3 to promote JNK3 phosphorylation was demonstrated by several groups using cell-based assays (Miller et al. 2001; Song et al. 2009; Seo et al. 2011) and in vitro reconstitution with purified proteins (Zhan et al. 2011b, 2013). Arrestin-3 is ubiquitously expressed (Attramadal et al. 1992; Sterne-Marr et al. 1993; Gurevich and Gurevich 2006a), whereas JNK3, which was first reported to be activated in arrestin-3-dependent manner, has more limited distribution, being expressed predominantly

in neurons, heart, and testes (Gupta et al. 1996; Davis 2000). In contrast, different isoforms of JNK1 and JNK2 are expressed as ubiquitously as arrestin-3 (Gupta et al. 1996; Davis 2000). Even though JNK3 has unique extended N terminus of 38 amino acids that was reported to be the main arrestin-3-binding site (Guo and Whitmarsh 2008; Song et al. 2009), high level of sequence conservation among JNK isoforms raises the question whether arrestin-3 regulates the activity of other JNK family kinases. Twelve main isoforms of JNK are generated by alternative splicing of three genes (Gupta et al. 1996; Davis 2000). The analysis of the knockout of individual JNK genes in mice revealed that different isoforms have distinct, although partially overlapping functions (Yang et al. 1997; Tournier et al. 2000, 2001; Kuan et al. 2003; Hunot et al. 2004). To test whether arrestin-3 is involved in the regulation of JNK1/2 isoforms, direct interaction between JNK1/2 and arrestin-3 was probed using purified protein in vitro (Kook et al. 2013). These experiments showed that the amount of arrestin-3 retained by His-tagged JNK2α2 is similar to that retained by His-tagged JNK3α2, whereas JNK1α1 shows a weaker binding to arrestin-3 in this paradigm (Kook et al. 2013). These in vitro binding data were supported by co-immunoprecipitation of arrestin-3 with these JNKs from cells, suggesting that arrestin-3 might be involved in the regulation of ubiquitous JNK isoforms. Next, MKK4/7–JNK1α1/JNK2α2 signaling modules were reconstructed with pure proteins in vitro with and without arrestin-3. These experiments revealed that the phosphorylation of JNK1/2 by both MKK4 and MKK7 is enhanced in the presence of an optimal arrestin-3 concentration (Kook et al. 2013), similar to the effect of purified arrestin-3 on JNK3α2 phosphorylation by MKK4/7 (Zhan et al. 2011b, 2013). Importantly, the biphasic dependence of JNK1α1/JNK2α2 phosphorylation by either MKK4 or MKK7 on arrestin-3 concentration in vitro suggests that arrestin-3 acts as a scaffold for MKK4/7-JNK1/2 signaling modules acting by bringing the two kinases to each other (Levchenko et al. 2000, 2004). Usually, it is hard to demonstrate the biphasic effect of scaffolding protein in intact cells, but in this case, it was shown that the activation of several isoforms of endogenous JNK1/2 by ASK1, MKK4, and MKK7 increases with the arrestin-3 expression up to a point, whereupon it is reduced by higher expression levels (Kook et al. 2013). These findings demonstrate that arrestin-3 promotes the activation of several isoforms of all three types of JNK kinases and can positively or negatively regulate JNK signaling in the majority of cell types.

9 Prospects of Manipulation of Cell Death and Survival by Signaling-Biased Arrestins

Despite remarkable recent progress in our understanding of arrestin-3 role in JNK activation (Zhan et al. 2011b, 2013; Kook et al. 2013), it remains to be elucidated how arrestin-3-dependent regulation affects cellular responses mediated by JNK signaling. JNK pathways regulate many vital cellular processes, including cell

death and survival (Davis 2000; Tournier et al. 2000; Weston and Davis 2007). In particular, JNK3 activation is believed to play a key role in triggering cell death, thereby participating in the pathogenesis of several human neurodegenerative disorders (Hunot et al. 2004; Morfini et al. 2009; Yoon et al. 2013). Neurons derived from JNK3-deficient mice are more resistant to Aβ-induced apoptosis than neurons from wild-type mice (Morishima et al. 2001), and JNK phosphorylation (which reflects activity) in human postmortem brains from Alzheimer's disease patients is markedly increased (Zhu et al. 2001). These reports suggest that modulation of JNK3 activation in Alzheimer's disease is a possible therapeutic target. Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra. Increased activation of the downstream target of JNK3, transcription factor c-Jun, is detected in mice treated with the neurotoxin 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine (MPTP) that specifically destroys dopaminergic neurons (Hunot et al. 2004) and in human postmortem brains from PD patients (Hunot et al. 2004). Mice deficient in JNK2 and JNK3 are more resistant to MPTP-induced injury than WT littermates, whereas JNK1 knockout mice show the same susceptibility to MPTP as controls (Hunot et al. 2004). Single JNK3 knockout mice also show neuroprotection against brain injury after cerebral ischemia-hypoxia (Kuan et al. 2003) and against excitotoxicity of the glutamate receptor agonist kainic acid (Yang et al. 1997). This evidence suggests that the inhibition of JNK activity, particularly the activity of JNK3, is a promising therapeutic approach to the treatment of neurodegenerative diseases. Several research laboratories have pursued small molecule inhibitors of JNK for therapeutic purposes, but most of the inhibitors found so far are not sufficiently specific, as they inhibit other kinases besides JNKs (Bennett et al. 2001; Scapin et al. 2003; Carboni et al. 2004; Resnick and Fennell 2004; Wang et al. 2004; Sabapathy 2012) A cell-permeable peptide inhibitor containing a 21-amino acid element of JIP-1 that interacts with JNKs (Barr et al. 2002) protects against cerebral ischemic injury in rodent model (Borsello et al. 2003). This example strongly supports the idea that molecular tools based on modified proteins, such as arrestin-3 mutant that acts as a dominant-negative silent scaffold (Breitman et al. 2012), have therapeutic potential as negative regulators of proapoptotic JNK signaling.

Considering how many diverse cellular functions are controlled by JNKs, one of the major issues that need to be resolved to enable therapeutic manipulation of JNK signaling is how to ensure high specificity of JNK modulation and to avoid affecting multiple processes. Catalytic domains of all JNK isoforms of each subfamily are identical, and even in the three JNK subfamilies, they are highly homologous (Bogoyevitch and Kobe 2006), which suggests that it is virtually impossible to achieve specificity by targeting ATP-binding site or other elements of the catalytic domain with small molecules. In contrast, different JNKs have distinct regulatory elements on their N- and C-termini, which mediate their interactions with upstream kinases, substrates, and scaffolding proteins. Scaffolds are important spatial and temporal regulators of JNK signaling. Scaffolds with distinct subcellular distribution and the ability to bind selected JNK isoforms, which can be either productive or

silent, have potential to enhance or inhibit JNK signaling in a specific cellular compartment to regulate distinct cellular responses. Productive scaffolds facilitate signaling by assembling individual components of MAPK cascade in correct orientation. However, supraoptimal levels of even productive scaffolds facilitate the formation of incomplete complexes, thereby blocking the signaling by sequestering these components away from potentially productive alternative signaling complexes, as was first suggested by mathematical modeling (Levchenko et al. 2000, 2004) and then recently shown experimentally (Zhan et al. 2011b, 2013; Kook et al. 2013). The identification of distinct binding sites for different JNK isoforms on arrestin-3 paves the way to designing small peptide inhibitors of the activation of individual JNKs. Signaling-biased arrestin-3 mutants, such as arrestin-3-KNC (Breitman et al. 2012) or other forms deficient in JNK activation (Seo et al. 2011), can be used to inhibit JNK signaling in cells. Moreover, arrestin-3- and other protein-based tools can be equipped with sequences that target them to specific subcellular compartments, such as cytosol or nucleus (Scott et al. 2002; Wang et al. 2003; Song et al. 2006). The construction of mutant scaffolds with signaling capability biased towards or against specific signaling pathways will allow more subtle and targeted modulation of MAPK pathways. This strategy can be used to correct signaling dysregulated in different pathological states, as well as to channel the signaling to pathways that have therapeutic potential even in cases where other molecular errors underlie the pathology.

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Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences

Charles Chavkin, Selena S. Schattauer, and Jamie R. Levin

Contents

Abstract Studies of kappa opioid receptor signaling mechanisms during the last decade have demonstrated that agonist activation of the receptor results in Gβγ-dependent signaling and distinct arrestin-dependent signaling events. Gβγ-dependent signaling results in ion channel regulation causing neuronal inhibition, inhibition of transmitter release, and subsequent analgesic responses. In contrast, arrestin-dependent signaling events result in p38 MAPK activation and subsequent dysphoric and proaddictive behavioral responses. Resolution of these two branches of signaling cascades has enabled strategies designed to identify pathway-selective drugs that may have unique therapeutic utilities.

Keywords Kappa opioid receptor • Dynorphin • Arrestin • p38 MAPK

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Abbreviations

ASK1	Apoptosis signal-regulating kinase 1
β2AR	β_2-Adrenergic receptor
CRF	Corticotropin-releasing factor
JNK	c-Jun N-terminal Kinase
ERK1/2	Extracellular signal-regulated kinase
GRK	G-protein receptor kinase
GPCRs	G-protein-coupled receptors
GIRK, Kir3	G-protein-gated inwardly rectifying potassium channel
GFAP	Glial fibrillary acidic protein
KOR	Kappa opioid receptor
rKOR	Rodent KOR
hKOR	Human KOR
MAPK	Mitogen-activated protein kinase
MAP3K5	Mitogen-activated protein kinase kinase kinase 5
PKC	Protein kinase C
5HT	Serotonin, 5-hydroxytryptamine

1 Introduction

The concept that arrestin association with G-protein-coupled receptors (GPCRs) does more than cause homologous receptor desensitization grew out of the realization that arrestin recruitment by the β_2-adrenergic receptor (β2AR) resulted in Src tyrosine kinase activation and phosphorylation of extracellular signal-regulated kinase (ERK1/2) (Daaka et al. 1997; Luttrell et al. 1999). Subsequently, Miller et al. (2003) found that the cytokine receptor US28 (a GPCR encoded by the human cytomegalovirus) activates p38 mitogen-activated protein kinase (MAPK) through a G-protein receptor kinase (GRK) and arrestin-dependent mechanism. In addition, Sun et al. (2002) found that the chemotaxic response of HeLa and HEK cells to cytokines mediated by the CXCR4 receptor also required p38 MAPK activation through receptor phosphorylation and arrestin-3 recruitment. Parallel studies showed that the GPCR-arrestin signaling complex activates c-Jun N-terminal kinase (JNK), also through a physical scaffolding mechanism (McDonald et al. 2000; Breitman et al. 2012). These observations lead to the concept that the arrestins can form a scaffold that physically links the GPCR to the three different MAPK signaling cascades: ERK1/2, p38 MAPK, and JNK (Burack and Shaw 2000; Pearson et al. 2001; DeWire et al. 2007).

The steps linking arrestin activation to p38 MAPK phosphorylation have not been fully visualized, but a requirement for apoptosis signal-regulating kinase 1 (ASK1), also known as mitogen-activated protein kinase kinase kinase 5 (MAP3K5), was suggested by the ability of dominant-negative mutant of ASK1 to block p38 MAPK activation (Sun et al. 2002). A plausible model for p38

activation suggests that activated arrestin forms a scaffold containing the required sequential cascade of the three kinases typically involved in a MAPK activation: a MAPKKK (possibly ASK1) activating a MAPKK (possibly MEK3 or MEK6), which in turn activates p38 MAPK (Burack and Shaw 2000; Pearson et al. 2001; Dewire et al. 2007). Presumably, arrestin association with the GRK-phosphorylated GPCR induces a structural rearrangement within arrestin–kinase complex, thereby facilitating the sequential phosphorylation reactions. However, the details of this cascade and differences between the cascades in different cell types and subcellular compartments have not yet been resolved.

Arrestin-dependent p38 MAPK activation results in a range of cellular and behavioral responses. In addition to mediating ***chemotaxic*** responses to cytokines (Sun et al. 2002), activation of p38 MAPK via arrestin association regulates ***apoptosis*** in mouse embryonic fibroblasts (Yang et al. 2012) and mediates endothelin-induced cell migration of mouse aortic smooth muscle cells (Morris et al. 2012). Arrestin-mediated p38 activation also induces ***hypertrophy*** and ***proliferation*** of GFAP-immunoreactive astrocytes in the spinal cord and brain (Bruchas et al. 2006; Xu et al. 2007). In addition, we recently found that arrestin-dependent p38 activation plays a key role in the ***behavioral stress response***, and it is the cellular details of this signaling cascade that we would like to summarize in this chapter.

In essence, our studies in mice have shown that:

1. Corticotropin-releasing factor (CRF) is released in the brain and hypothalamus in response to stress exposure.
2. CRF acts broadly in brain to coordinate the physiological, adaptive response to stress necessary for survival.
3. One of the cellular responses to CRF is the stimulated release of the endogenous dynorphin opioid peptides (Land et al. 2008; Bruchas et al., 2009).
4. Dynorphins selectively activate the kappa opioid receptors (KOR), which are Gi/o-coupled GPCRs (Chavkin et al. 1982; Bruchas et al. 2007a, b; Land et al. 2008; Bruchas et al. 2011; Lemos et al. 2012).
5. Sustained KOR activation results in GRK3-mediated phosphorylation of Ser369 in rodent KOR and subsequent arrestin-3 recruitment (Bruchas et al. 2006).
6. The KOR–arrestin complex initiates the phosphorylation and activation of p38α MAPK at multiple sites within the brain (Bruchas et al 2011; Lemos et al. 2012; Schindler et al. 2012).
7. p38α activation at one of these sites (the nerve terminals of the serotonergic neurons projecting from the dorsal raphe nucleus to the ventral striatum) causes the translocation of the serotonin transporter (SERT; SLC6A4) from an endosomal compartment to the nerve terminal surface (Bruchas et al. 2011; Schindler et al. 2012).
8. Increase in surface expression of SERT pumps serotonin (5HT) back into the nerve terminal more efficiently and thereby produces a transient hyposerotonergic state in the ventral striatum (Bruchas et al. 2011; Schindler et al. 2012).

9. The reduction in 5HT tone in the ventral striatum contributes to the stress-induced dysphoria evident as behavioral aversion in the mice exposed to the stressful experience (Land et al. 2008; Bruchas et al. 2011; Schindler et al. 2012).

Evidence supporting this proposed cascade is summarized below.

2 Kappa Opioid Receptors

Kappa opioid receptors are members of the Gi/o-coupled superfamily of G-protein-coupled receptors (Bruchas and Chavkin 2010). Consistent with the data concerning other members of this class, kappa receptor activation results in a broad range of signaling events including membrane-delimited Gβγ-mediated regulation of calcium and potassium conductances. Gβγ released by kappa receptor activation increases G-protein-gated inwardly rectifying potassium channel (Kir3) activation and positively shifts the activation threshold of voltage-sensitive calcium channels (VSCC), thereby inhibiting calcium conductance (Werz and Macdonald 1984; Cherubini and North 1985; Herlitze et al. 1996). The net effect of these membrane-delimited effects on ion conductance is to reduce somatic excitability and calcium influx at the nerve terminal; kappa receptor activation has been shown to presynaptically inhibit the release of a broad range of neurotransmitters through these ionic mechanisms (Grudt and Williams 1995; Simmons and Chavkin 1996).

G-protein stimulation by kappa receptors also activates a variety of kinases including ERK1/2, JNKs, PKC, and p38 MAPKs in receptor-transfected cells, primary cultures of neurons and astrocytes, and in kappa opioid receptor expressing neurons in the brain (see Bruchas and Chavkin 2010). These activated kinases phosphorylate a variety of substrates, including transcription factors to regulate gene expression and various cytoplasmic proteins to affect neuronal physiology (to be described further below).

Again, like other G-protein-coupled receptors, agonist-activated kappa receptors are substrates for GRKs, which phosphorylate specific serine residues in the carboxy-terminal domain of the receptor [i.e., serine-369 in the rodent kappa receptor (rKOR) and the homologous residue serine-358 in the human kappa receptor (hKOR) sequence] (Appleyard et al. 1999; Li et al. 2002; McLaughlin et al. 2003; Schattauer et al. 2012). Arrestin binding to the GRK-phosphorylated kappa receptor sterically inhibits further G-protein activation and results in homologous desensitization of membrane-delimited signaling, but arrestin association is required for the late phase of ERK1/2 activation (Bruchas et al. 2008; McLennan et al. 2008) and for p38 MAPK activation by kappa receptors (Bruchas et al. 2006; Xu et al. 2007). Thus, arrestin binding to kappa receptors shifts agonist signaling from membrane-delimited pathways to alternative effector pathways.

With this wide range of possible cellular signaling responses, it should not be surprising that the ability of a kappa agonist to activate one pathway (its efficacy)

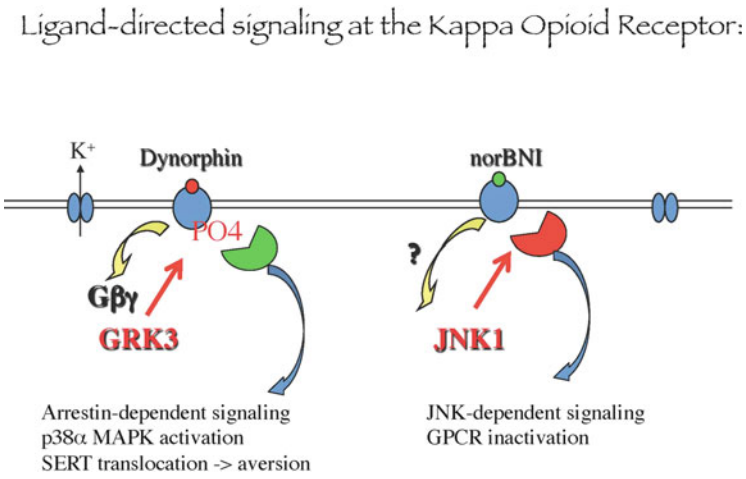

Fig. 1 Ligand-directed signaling differences between kappa opioids. Strong agonists, like dynorphin, activate kappa receptors to stimulate Gβγ-dependent responses including presynaptic inhibition of transmitter release (calcium channel inhibition) and somatic membrane hyperpolarization (potassium channel activation). Sustained kappa receptor activation results in the phosphorylation of specific serine residues in the carboxy-terminal domain of the receptor and subsequent arrestin (green symbol) recruitment. The resulting arrestin activation enables p38 MAPK activation (phosphorylation), and the cellular consequences include astrocyte activation, Kir3 potassium channel phosphorylation (and deactivation), and SERT translocation. At the behavioral level, presynaptic inhibition of transmitter release underlies the analgesic responses, and SERT translocation mediates dysphoria and proaddictive responses. In contrast, long-acting kappa antagonists, like norBNI, cause receptor inactivation through a c-Jun N-terminal kinase (JNK)-dependent mechanism without stimulating Gβγ-dependent responses

does not need to be the same as for all the different pathways. Ligand-directed signaling differences have been documented in other GPCR systems (Urban et al. 2007) (see Chap. 3). Based on these insights, kappa ligands can be conceptually divided into (1) ***strong agonists*** (able to activate all of the Gβγ- and arrestin-dependent signaling events), (2) ***weak agonists*** (able to activate Gβγ-, but not arrestin-dependent signaling events), (3) ***neutral antagonists*** (that bind receptor but do not evoke any signaling responses), and (4) ***collateral agonists*** (that bind to kappa receptors to activate one of the alternative signaling pathways without activating Gβγ-dependent responses) (Fig. 1). ***Arrestin-biased agonists*** (see Chap. 3) at the kappa receptor that activate arrestin-dependent signaling without efficiently activating Gβγ signaling could be postulated by analogy to the parathyroid hormone and angiotensin II receptors (Gesty-Palmer et al. 2009; Violin et al. 2010); however, examples of this type of ligand have not yet been characterized.

Dynorphin peptides, salvinorin A, U50,488, U69,593, and enadoline are prominent members of the first category. Buprenorphine, naloxone, and naltrexone are examples of neutral antagonists (although they lack kappa receptor selectivity). A ligand that activated Gβγ signaling but did not efficiently stimulate GRK would be

expected to activate the membrane-delimited signaling but not the arrestin-dependent responses (Chavkin 2011). 6′GNTI has been suggested as an example of a G-protein-biased kappa receptor agonist that does not recruit arrestin (Rives et al. 2012). An example of this type of pathway-selective ligand in a different receptor system is morphine, which is a strong opioid analgesic acting through mu opioid receptors, but does not efficiently activate arrestin-dependent responses (Dang and Christie 2012). norBNI and JDTic are examples of the latter category; the selective kappa ligands norBNI and JDTic do not activate Gβγ- or arrestin-dependent pathways, but do effectively activate JNK pathways upon kappa receptor binding (Bruchas et al. 2007a, b; Melief et al. 2010).

3 Stress-Induced Release of Dynorphin Increases Phospho-p38 MAPK in a GRK3- and Arrestin-3-Dependent Manner

Efforts to understand opioid receptor tolerance mechanisms entered a new molecular biology phase after the delta opioid receptors were cloned by Kieffer and Evans in 1994, and the mu and kappa sequences were deduced shortly afterwards (Akil et al 1996). In a series of site-directed mutagenesis studies using *Xenopus* oocyte expression, the serine-369 residue in the carboxy-terminal domain was found to be the critical GRK phosphorylation site required for homologous rKOR desensitization (Appleyard et al. 1999). To determine if this phosphorylation event also regulated kappa opioid signaling in vivo, we generated a phospho-selective antibody, KOR-p, that could distinguish phosphorylated KOR-pSer369 from the unphosphorylated receptor (McLaughlin et al. 2003). Importantly, the increase in KOR-p immunoreactivity induced by the kappa agonist U50,488 was not evident in GRK3−/− mice. The selective role of GRK3 (without compensation by other GRK isoforms) was a surprise. Mice lacking GRK3 showed reduced analgesic tolerance to U50,488 (McLaughlin et al. 2003).

The high degree of cellular resolution of the immunohistochemical KOR-p staining provided a new opportunity to detect sites of dynorphin action in the brain, and we next adopted a partial sciatic nerve ligation method previously shown by Porecca and colleagues to evoke endogenous dynorphin release (Wang et al. 2001). We found that KOR-p immunoreactivity was increased in the spinal cord following partial sciatic nerve ligation in wild type, but not in KOR−/−, prodynorphin−/−, or GRK3−/− mice (Xu et al. 2004). Sustained dynorphin release following nerve ligation produced tolerance to the analgesic effects of U50,488, but nerve ligation did not produce tolerance in GRK3−/− or prodynorphin −/− mice (Xu et al. 2004). These results established that kappa opioid receptor desensitization occurred both in vivo and in vitro through a GRK-/arrestin-dependent mechanism.

4 Astrocyte Activation by Dynorphin Occurs Through an Arrestin/p38 MAPK Mechanism

One of the striking features of nerve ligation is that it causes the robust activation of astrocytes, as documented by the increase in number of GFAP-immunoreactive cells in the spinal cord. However, we were surprised to observe that the increased GFAP immunoreactivity was not evident in prodynorphin−/− or GRK3−/− mice and that the activation of astrocytes by nerve ligation could be blocked by the p38 MAPK inhibitor SB 203580 (Xu et al. 2007). Using KOR-transfected AtT20 cells, we found that kappa receptor stimulation increased phospho-p38 immunoreactivity and that the increase could be blocked by a dominant-negative form of arrestin but not evident if kappa receptor phosphorylation was blocked by alanine substitution for Ser369 in KOR (Bruchas et al. 2006). A GRK3-/arrestin-dependent mechanism of p38 activation in astrocytes stimulated by kappa agonists in vivo and in vitro was also documented by confocal imaging and Western blot analysis (Bruchas et al. 2006; Xu et al. 2007). p38 MAPK activation was not evident in ether striatal astrocytes or neurons isolated from KOR−/− or GRK3−/− mice, and cultured striatal astrocytes pretreated with siRNA for arrestin-3 were also unable to activate p38 in response to U50,488 treatment (Bruchas et al. 2006). McLennan et al. (2008) also found that proliferation of immortalized astrocytes in culture could be stimulated by kappa opioids in a Gβγ- and arrestin-dependent manner. They attributed these effects to pERK activation—not p38; however, in a subsequent study, they reported that both ERK and p38 pathways stimulated oligodendrogenesis in a similar culture system (Hahn et al. 2010). Extending these findings, we found that forced swim stress also activates GFAP-immunoreactive astrocytes in hippocampus and cortex by stimulating this dynorphin-KOR-GRK3-arrestin $\Rightarrow$ phospho-p38 MAPK cascade (Messinger and Chavkin unpublished observations).

5 Kappa Receptor Activation of Arrestin/p38 MAPK Regulates the Potassium Channel Kir3

Prior studies showed that tyrosine phosphorylation in the N-terminal cytoplasmic domain of the G-protein-gated inwardly rectifying potassium channel, Kir3.1, facilitates channel deactivation by increasing the intrinsic GTPase activity of the channel (Ippolito et al. 2002, 2005). Dynorphin released during forced swim stress or following sciatic nerve ligation also resulted in tyrosine phosphorylation of Kir3.1 at these regulatory residues (Clayton et al. 2009). Channel phosphorylation in the dorsal horn of the spinal cord of nerve-ligated mice required GRK3 phosphorylation of the kappa opioid receptor, arrestin recruitment, and subsequent p38 MAPK activation (Clayton et al. 2009). Whole cell voltage clamp of AtT20 cells expressing kappa receptors demonstrated that p38 activation reduced the potassium current through a Src kinase-dependent mechanism; the enhanced channel

deactivation could be blocked by the Src inhibitor PP2. Similar mechanisms also regulate Kir3 current in serotonergic neurons of the dorsal raphe nucleus in the brain (Lemos et al. 2012). Acute activation of kappa receptors in these neurons increases potassium conductance through G-protein-gated inwardly rectifying channel, but sustained kappa receptor activation by repeated stress exposure causes channel phosphorylation and subsequent channel inactivation through the arrestin-dependent p38 MAPK mechanism (Lemos et al. 2012).

6 Kappa Receptor Activation of Arrestin/p38 MAPK Activates the Serotonin Transporter

Selective kappa agonists produce feelings of dysphoria in humans and aversion responses in experimental animals (Pfeiffer et al. 1986; Shippenberg and Herz 1986). Stress-induced release of the endogenous dynorphin opioid peptides selectively activates kappa opioid receptors and produces dysphoria in experimental animals (McLaughlin et al. 2006; Bruchas et al. 2007a, b; Land et al. 2008). The dysphoria caused by stress-induced activation of the dynorphin-kappa opioid systems results in a potentiation of the rewarding valence of cocaine and reinstatement of extinguished cocaine drug seeking, which may help explain how stress increases the risk of drug addiction.

We used a conditional gene deletion approach to define the molecular events responsible for these behavioral responses. Using mice having lox-p excision sequences flanking the p38α MAPK, we found that selective inactivation of p38 signaling in serotonergic neurons of the dorsal raphe nucleus blocked defeat-induced social aversion and stress-induced reinstatement of cocaine place preference (Bruchas et al. 2011). In addition, selective excision of p38α MAPK in serotonergic neurons blocked stress-induced potentiation of cocaine place preference (Schindler et al. 2012). These behavioral responses were each caused by stress-induced dynorphin release, kappa opioid receptor activation, GRK3-dependent kappa receptor phosphorylation, and subsequent arrestin recruitment and activation. Previous reports had demonstrated a role for p38 MAPK in the modulation of the plasma membrane serotonin transporter (SERT, SLC6A4) function in vitro (Zhu et al. 2004, 2005; Samuvel et al. 2005), and using a cell-surface biotinylation and Michaelis-Menten kinetic analysis of 5HT transport, we found that stress-induced activation of p38α in serotonergic neurons causes SERT translocation from a cytoplasmic endosomal compartment to the cell surface (Bruchas et al. 2011; Schindler et al. 2012). Although the dorsal raphe sends afferent projections broadly throughout the forebrain, dynorphin-dependent SERT translocation was evident only in the serotonergic projection to the ventral striatum (Schindler et al. 2012). These findings suggest that stress-induced dysphoria mediated by arrestin/p38 MAPK activation is caused by a transient hyposerotonergic state in the nucleus accumbens (Fig. 2).

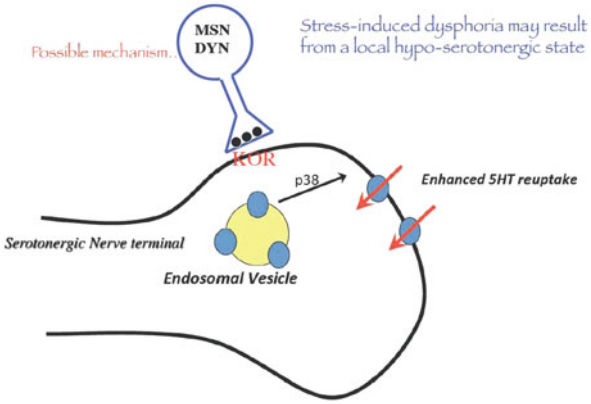

Fig. 2 Stress exposure regulates serotonergic neurotransmission. Dynorphin opioid peptide release from medium spiny neurons (MSN) in the ventral striatum activates kappa opioid receptors (KOR) expressed on the terminals of the serotonergic neurons innervating the nucleus accumbens. Through a G-protein receptor kinase 3 (GRK3)-dependent mechanism, arrestin activates p38α MAPK in the nerve terminals, thereby increasing cell-surface expression of the serotonin transporter. The transient hyposerotonergic state in the nucleus accumbens likely contributes to the dysphoria underlying stress-induced aversion and stress-induced potentiation of the rewarding valence of abused drugs

7 Conclusions

These studies show that kappa receptor activation by either endogenous dynorphin release in vivo or pharmacological activation in vitro causes p38 MAPK activation through a GRK3- and arrestin-dependent mechanism. p38 MAPK is likely to have a broad range of substrates and to regulate a diverse group of processes. Several of these have been identified, but others are plausible. Several important questions have not been resolved, including the characterization of specific signaling steps linking kappa receptor activation of arrestin to p38 MAPK activation and differences in signaling in different cell types and subcellular compartments. Since arrestin/p38 signaling is essential for the dysphoric and proaddictive effects of kappa opioids, but not for their analgesic effects, we expect that pathway-selective kappa agonists, which need to be identified, will have therapeutic advantages.

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Arrestin-Dependent Localization of Phosphodiesterases

Miranda J. Willis and George S. Baillie

Contents

Abstract Many G-protein-coupled receptors trigger the synthesis of cAMP in order to transduce signals from the membrane into the cell cytoplasm. As stimulation of each receptor type results in a specific physiological outcome, compartmentalization of proteins that make, break, and are activated by cAMP underpin receptor-specific responses. Until 2002, it was thought that static compartmentalization of phosphodiesterase 4 (PDE4), conferred by N-terminal targeting sequences, was one way to shape intricate cAMP gradients that formed after receptor activation. Discovery of the PDE4–β-arrestin complex represented a major breakthrough in cAMP signaling, as it spurred the initial realization that PDE4s could be transported to sites of high cAMP to orchestrate destruction of the second messenger at the same time as the receptor's signal to the G-protein is silenced. This chapter charts the scientific process that led to the discovery and characterization of the PDE4–β-arrestin interaction and discusses the known functions of this signaling complex.

Keywords Phosphodiesterase type 4 (PDE4) • Cyclic AMP • Compartmentalization • Peptide array • Protein kinase A (PKA) • β-arrestin

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1 Phosphodiesterases Underpin Compartmentalized cAMP Responses

Cell surface receptors sense the extracellular environment and react to chemical cues such as hormones, neurotransmitters, or signaling peptides by stimulating the creation of "second messengers" that act to trigger intracellular cell signaling cascades. One such second messenger, cyclic AMP (cAMP), is produced by adenylyl cyclase (Cooper and Crossthwaite 2006) and acts via one of three known effector proteins: cAMP-dependent protein kinase [also known as protein kinase A (PKA)] (Taylor et al. 2012), cyclic nucleotide gated ion channels (Kaupp and Seifert 2002), or the guanine-nucleotide exchange factor (GEF) known as the exchange protein activated by cAMP (EPAC) (Bos 2006). As many receptor types utilize cAMP as a second messenger but manage to transduce signals that result in a highly specified cellular response, it is logical to propose that cAMP responses are highly compartmentalized. This concept was pioneered by Brunton and colleagues in the early 1980s (Brunton et al. 1981) and has been upheld by recent experimentation using probes that detect real-time, spatial, and temporal changes in intracellular cAMP concentrations following receptor activation [reviewed in Edwards et al. (2012)]. One major finding from these studies is that the compartmentalization of cAMP responses is underpinned by the action of the only known superfamily of enzymes that degrade cAMP, phosphodiesterases (PDEs). PDEs are divided into 11 families [reviewed in Conti and Beavo (2007)] with PDE4, PDE7, and PDE8 being cAMP specific, PDE5, PDE6, and PDE9 being cGMP specific, and the other 5 PDEs (PDE1, PDE2, PDE3, PDE10, and PDE11) having dual specificity with differing affinities for both types of cyclic nucleotides. Recently, there has been much interest in the PDE4 family, as a PDE4 inhibitor (roflumilast) has been licensed in Europe and the USA for the treatment of severe chronic obstructive pulmonary disease (COPD) (Fabbri et al. 2010). PDE4s are encoded by 4 genes (A, B, C, and D) and these give rise to at least 25 different proteins (6 PDE4A forms, 5 PDE4B forms, 3 PDE4C forms, and 11 PDE4D forms) via mRNA splicing and promoter diversity (Houslay et al. 2005). Indeed, study of this PDE family has provided the paradigm for intracellular targeting of cAMP hydrolysis and this is because all PDE4 isoforms have similar Kms and Vmax for cAMP hydrolysis; hence their functional role is determined largely by their cellular location, interaction with other signaling proteins, and posttranslational modifications (Baillie 2009). In keeping with all other PDE families, PDE4s have a complex modular structure, consisting of a conserved catalytic domain, subfamily-specific C-terminal domain, dual regulatory domains called upstream conserved region 1 (UCR1), and upstream conserved region 2 (UCR2) together with an isoform-specific N-terminal region (Houslay et al. 2005). Importantly, the fundamental roles that individual PDE4 isoforms play in tailoring compartmentalized cAMP signals are conferred by the unique localization sequences that are contained within isoform-specific N-terminal regions. This "postcode" sequence or other localization sequences direct the association of PDE4 family members to a variety of inert scaffolds,

lipids, or active proteins. These include AKAP18 (Stefan et al. 2007), RACK1 (Bolger et al. 2006), mAKAP (Dodge-Kafka et al. 2005), β1-adrenergic receptor (Richter et al. 2008), immunophilin XAP2 (Bolger et al. 2003b), SRC family tyrosine kinase (Beard et al. 1999), the p75 neurotrophin receptor (Sachs et al. 2007), the dynein complex member Nudel (Collins et al. 2008), disrupted in schizophrenia 1 (DISC1)(Millar et al. 2005), the cardiac IKs potassium channel (Terrenoire et al. 2009), phosphatidic acid (Grange et al. 2000; Baillie et al. 2002), the PDZ domain containing protein Shank2 (Lee et al. 2007), the small heat-shock protein 20 (HSP20) (Edwards et al. 2011; Sin et al. 2011), and the signaling scaffold protein β-arrestin (Perry et al. 2002). It is the latter interaction that will be the focus of this review.

2 Discovery of the Interaction Between PDE4 Enzymes and β-Arrestin

Work by the Lefkowitz lab into mechanisms that aid desensitization of Gs-coupled receptors noted that the kinetics of β-arrestin recruitment to the membrane closely matched that of PDE4 (Perry et al. 2002). A similar result was observed when translocation of both proteins to purified fractions containing the β2-adrenergic receptor (β2AR) was monitored. Follow-up work showed that β-arrestin 1 and β-arrestin 2 could actually form a complex with five members of the PDE4D subfamily, suggesting that the association between the β-arrestin and PDE4s was conferred by a region that was common to all PDE4 enzymes (Perry et al. 2002). Realization of the functional significance of the transient translocation of a PDE4–β-arrestin complex following β2AR stimulation was achieved following experimentation with a dominant negative, catalytically dead form of PDE4D5 (Baillie et al. 2003). This mutant could associate with β-arrestin to effectively displace the endogenous active PDE4 from that location within the cell. Disruption of the pool of PDE4 associated with β-arrestin increased localized PKA phosphorylation of the β2-AR (Lynch et al. 2005) and attenuated the reprogramming of its coupling specificity from Gs to Gi (Baillie et al. 2003) dampening the mitogenic signal mediated by the tyrosine kinase Src (Daaka et al. 1997). Such an action is in synergy with the primary function of β-arrestin, which is to sterically hinder signaling between receptors and Gs, initiating a reduction in adenylyl cyclase activation and a subsequent decrease in cAMP production (Shenoy and Lefkowitz 2011). In effect, the β-arrestin–PDE4D5 complex serves to orchestrate a dual desensitization process where the cAMP "message" is destroyed at the same time as the receptor's signal to the G-protein is silenced. This in turn effectively "resets" the receptor for another round of agonist challenge (Baillie and Houslay 2005). The discovery of the β-arrestin–PDE4 complex represented a major breakthrough in cAMP signaling as it (a) provided the first evidence for a specific phenotypic function that could be ascribed to a single PDE4 isoform type and (b) spurred the initial realization that

although PDE4s are compartmentalized via targeting sequences within their N-terminal region, this did not mean they were static but instead could be transported in conjunction with other proteins to sites of high cAMP concentrations within the cell (Lynch et al. 2007).

3 Molecular Characterization of the PDE4–β-Arrestin Interaction

It has been established over many years that although PDE4-specific inhibitors developed to target the enzyme's active site have potential for the treatment of a variety of inflammatory (Dastidar et al. 2007), respiratory (Page and Spina 2012), and neurological diseases (O'Donnell and Zhang 2004), their clinical utility is compromised by mechanism-associated side effects that limit maximally tolerated doses [reviewed (Spina 2008)]. Headache, nausea, emesis, and diarrhea are the most commonly reported side effects and these stem from the inhibition of PDE4s in nontarget tissues. Specifically, PDE4D expression is high in an area of the brain known to trigger nausea called the area postrema (Miro et al. 2002) and PDE4 inhibition may also act directly on the gastrointestinal tract (Perez-Torres et al. 2000). Put simply, off-target effects of PDE4 inhibitors stem from the fact that the catalytic units of all PDE4 enzymes show a high degree of similarity in structure and sequence (Houslay et al. 2005) and so molecules that are directed at the PDE4 active site, to occlude cAMP, tend to have a similar affinity for all subtypes (on drawbacks of small molecule inhibitors, see Chap. 1). Clearly, alternative approaches that inhibit or displace discretely targeted pools of individual PDE4 isoforms are likely to have inherent advantages over PDE4 inhibitors that globally inhibit all PDE4 isoforms in all their various signaling complexes. The archetype PDE4 inhibitor, rolipram, has been of use for gaining insight into general functions of the PDE4 family, but because it inhibits all PDE4 isoforms, it has not had utility in understanding the unique, nonredundant roles that particular PDE4 isoforms play in shaping compartmentalized cAMP signaling (Baillie 2009). Such analysis has recently been possible for PDE4D5 using novel technologies such as dominant negative transfection (Baillie et al. 2003; McCahill et al. 2005) and targeted disruption of PDE4D5 complexes by cell-permeable peptides (Smith et al. 2007). These novel approaches have allowed insight into the function of PDE4D5 when sequestered to specific scaffolds, like β-arrestin. Additionally, siRNA silencing, which knocks down PDE4D5, has allowed insight into global actions of this isoform (Lynch et al. 2005; Willoughby et al. 2007). The dominant negative approach mentioned above relies on ectopically expressed forms of PDE4 that have been engineered, by a single point mutation deep within their catalytic site, to be incapable of hydrolyzing cAMP. Such overexpressed, exogenous forms act to displace the cognate endogenous species from their sites of anchor within cells to enable detection of functional significance of the replaced, anchored

species. This approach has provided essential clues to the function of targeted PDE4 isoforms, but it cannot differentiate between differently targeted subpopulations of the same isoform, hampering investigations of function for isoforms such as PDE4D5, which can have multiple, nonredundant roles within the same cell or tissue due to being targeted to different signalosomes.

A novel approach directed against the targeting of PDE4D5 to specific signalosomes, such as the scaffold protein, β-arrestin, has been facilitated by the introduction of peptide array technology. This technique not only allows rapid insight into the molecular nature of specific protein–protein interactions but also aids the rapid development of molecules able to disrupt specific partnerships, such as that between PDE4D5 and β-arrestin (Smith et al. 2007). Knowledge of the sequence of one of the interacting partners is used to generate a library of overlapping, spotted, immobilized 25-mer peptides that "scan" the target protein. This scanning peptide array library is then probed with the purified partner protein using a simple "far-western" overlay protocol. Positive spots containing putative binding sequences are further explored by substitution analysis, e.g., with alanine or aspartate, to generate a "bar code" of key residues implicated in binding. This approach has been used to map the binding sites for PDE4 on β-arrestin (Baillie et al. 2007) and β-arrestin on PDE4 (Bolger et al. 2006) (see below). Information gleaned from this technique can also be used to drive directed mutagenesis studies to formally map interaction sites in cellular proteins. The identified peptide sequences can be used to drive the rapid generation and optimization of powerful small peptides and related agents able to disrupt specific protein interactions for therapeutic development and for cell and in vivo studies aimed at attributing phenotypic outputs to specific signaling complexes.

Following analysis of the PDE4 complement that co-immunoprecipitated with β-arrestin from a variety of cell lines, it was discovered that although PDE4D5 generally accounted for between 15 and 50 % of the cellular PDE4D protein, PDE4D5 represented the vast majority (at least 80 %) of the pool that associated with β-arrestin (Bolger et al. 2003a). Truncation analysis highlighted the fact that there were two sites on PDE4D5 that mediated its interaction with β-arrestin. One was located in the common catalytic region of the enzyme and the second within the unique amino-terminal targeting region. The former result explains why multiple PDE4 isoforms show some association with the scaffold (Perry et al. 2002; Baillie et al. 2003), while the second explains why PDE4D5 is β-arrestin's PDE4 partner of choice (Bolger et al. 2003a). Careful mapping of the β-arrestin-binding sites on PDE4D5 using yeast two-hybrid analysis (Bolger et al. 2003a, 2006), peptide array (Bolger et al. 2006), NMR (Smith et al. 2007), and binding studies following mutagenesis or truncation (Bolger et al. 2003a, 2006) showed that β-arrestin interacted with an extended portion of the PDE4D5 N terminus spanning from N22 until T45 and also with a well-defined surface exposed patch on helix-17 within the catalytic domain. Surprisingly, in the same study, another scaffold protein, RACK1, was also shown to bind over the same region of the N terminus of PDE4D5 (using a separate set of specific amino acids compared with those utilized by β-arrestin). Competition to sequester PDE4D5 meant that RACK1 and

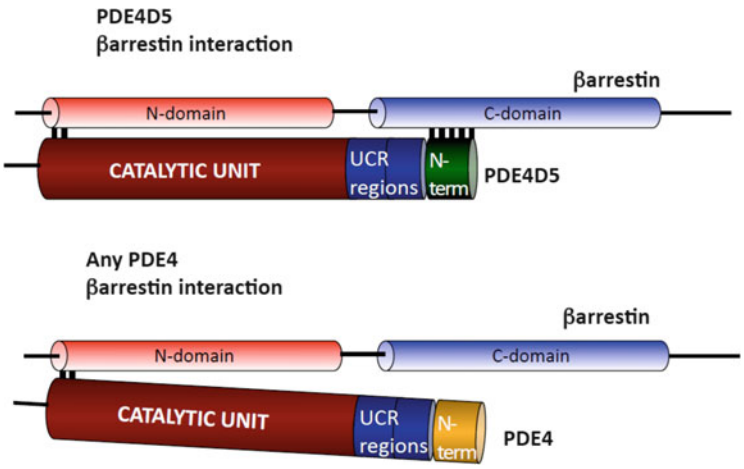

Fig. 1 Peptide array, yeast two-hybrid analysis, and site-directed mutagenesis delineate the sites of association between β-arrestin and PDE4 isoforms. PDE4D5 is the "preferred" partner as it associates at two main sites, one within its unique N terminus (N-term) and one at a common sequence in the catalytic domain that is conserved in all PDE4s. All PDE4s can associate with β-arrestin at a site within the scaffold's N-domain (*UCR* Upstream conserved regions)

β-arrestin associate with different "pools" of the enzyme, as their binding is mutually exclusive (Bolger et al. 2006).

Peptide array technology, yeast two-hybrid analysis, and site-directed mutagenesis have also been used to delineate the docking sites for PDE4s on β-arrestin (Baillie et al. 2007). In line with many other β-arrestin-binding partners (Song et al. 2009; Ahmed et al. 2011; Seo et al. 2011), PDE4s bind to regions within the N- and C-domains of β-arrestin. Specifically, the common catalytic site of all PDE4s binds to the β-arrestin N-domain and the 4D5 N-terminal regions associates directly with two regions in the β-arrestin C-domain (Fig. 1). Mutations in β-arrestin at these PDE4-binding sites did not affect recruitment of the scaffold to the β2AR but did result in a hyper-phosphorylation of the receptor by PKA following isoprenaline treatment (Baillie et al. 2007).

As mentioned above, with respect to PDE4D5, peptide array analysis has been used to define binding sites for both scaffolds, β-arrestin and RACK1 (Yarwood et al. 1999; Bolger et al. 2002, 2003a, 2006). This information has been used to design peptide agents that could disrupt PDE4D5's interaction with RACK1 or β-arrestin or both at the same time (Smith et al. 2007). Subsequent evaluation of cell-permeable versions of these peptide disruptors proved that the disturbance of targeted pools of the same isoform (in this case PDE4D5) could lead to very different functional outcomes. The β-arrestin–PDE4D5 disruptor, for example, attenuated recruitment of PDE4D5 to the β2-adrenergic receptor leading to a hyper-phosphorylation of the receptor after stimulation (Smith et al. 2007), whereas the RACK1–PDE4D5 disruptor was effective at preventing the formation of

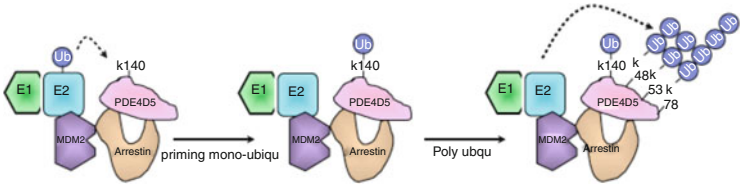

Fig. 2 β-Arrestin orchestrates the ubiquitination of PDE4D5 by scaffolding the MDM2 E3 ligase complex in close proximity to the PDE. A priming ubiquitination at K140 within the UCR1 region allows subsequent ubiquitination of three other sites within the N terminus of PDE4D5 (K48, K53, and K78). Ubiquitination of PDE4D5 in this manner increases the fidelity of interaction between the enzyme and β-arrestin and this in turn increases the efficiency of β2AR desensitization

spreading initiation centers in cancer cells (Serrels et al. 2010). Clearly, both peptides have potential as therapeutics, the former for the treatment of asthma where PDE4D5 is upregulated following chronic bronchodilator use (Le Jeune et al. 2002) (Fig. 3) and is a key regulator of β2-AR-induced cAMP turnover within human smooth muscle (Billington et al. 2008) (see below) and the latter as an agent to prevent polarization and metastasis of cancer cells (Serrels et al. 2010) via an EPAC signaling axis (Serrels et al. 2011).

Another factor affecting the interaction of β-arrestin and PDE4D5 is posttranslational modification by ubiquitin. Isoprenaline triggers the rapid and transient ubiquitination of PDE4D5 by the E3 ligase MDM2 (Li et al. 2009a). The protein complex containing MDM2 and PDE4D5 is held together by β-arrestin to allow ubiquitination of a site within the PDE4D5 UCR1 region (Fig. 2). This acts as a priming step for subsequent ubiquitination at three other sites within the unique N terminus of PDE4D5. Modification of the PDE in this manner elicits an increase in the fraction of PDE4D5 sequestered by β-arrestin, thereby contributing to the fidelity of the interaction. This in turn reduces the amount of PDE4D5 sequestered by other known PDE4D5-binding partners.

4 Involvement of the PDE4D5–β-Arrestin Complex in Asthma

In addition to the well-documented anti-inflammatory action of cAMP in inhibiting cell proliferation and secretion of inflammatory mediators (Torphy 1998), relaxation of airway smooth muscle is dependent on the elevation of cAMP and is targeted by frontline bronchodilators (see also Chap. 20). Most of these are β2-adrenergic receptor (β2AR) agonists and chronic use can lead to loss of bronchodilator effect, increased airway hyper-reactivity, and increased risk of asthma morbidity and mortality due to the masking of symptoms of deteriorating asthma and worsening asthma control (Beasley et al. 1999; Nelson et al. 2006). Development of airway

tolerance to β2-agonists is thought to have the most profound adverse effect on the asthmatic phenotype and results from a prolonged, agonist-specific desensitization of the receptor itself (Giembycz and Newton 2006). One mechanism that can contribute to desensitization, especially with respect to pulmonary β2AR signaling, is the upregulation of cAMP-degrading PDE4D5, which can be further activated by PKA-dependent phosphorylation (Giembycz 1996). Increases in PDE4 activity compromise Gs-coupled signaling, resulting in heterologous desensitization of events that promote airway relaxation. Indeed, there is much evidence to support this concept, as Gs-coupled receptor agonist-dependent increases in PDE4 expression have been noted in many cell types implicated in the pathogenesis of asthma (Torphy et al. 1995; Verghese et al. 1995; Seybold et al. 1998; Dasi et al. 2000). This phenomenon has also been noted in vivo, as PDE4 is upregulated in the lungs of rats chronically treated with salbutamol (Finney et al. 2000, 2001). Most of the PDE4 activity in vascular smooth muscle (VSM) and airway smooth muscle (ASM) is attributed to isoforms of the PDE4D subfamily (Nino et al. 2009) and studies in PDE4D-deficient mice report a fourfold increase in airway cAMP accumulation following Gs-coupled receptor activation (Hansen et al. 2000; Mehats et al. 2003). Notably, this increase in cAMP is associated with altered sensitivity to PGE2-induced relaxation of PDE4D−/− tracheas and suggests clinical relevance in the development of tolerance following long-term use of β2-adrenoceptor agonists.

Dynamic repositioning of a fraction of PDE4D5, by virtue of its association with the signal scaffold protein β-arrestin, allows recruitment of PDE activity to sites of high cAMP at the plasma membrane following agonist stimulation of the β2AR (Perry et al. 2002; Baillie et al. 2003). Upregulation of PDE4D5 in human ASM following prolonged cAMP signaling is now well documented and is thought to provide a negative feedback mechanism functionally downregulating the bronchodilator effect of β2-agonists (Le Jeune et al. 2002; Billington et al. 2008; Hu et al. 2008; Nino et al. 2009). The primary mechanisms behind the overexpression of PDE4D5 in human ASM following cAMP elevation depend on (1) a cAMP response element (CRE)-containing, isoform-specific promoter that drives de novo synthesis of the enzyme (Le Jeune et al. 2002) and (2) the induction of transcriptional regulation of PDE4D5 by ERK MAP kinases following Gi protein signaling triggered by βg-subunits (Nino et al. 2010; Hu et al. 2008; Nino et al. 2009). Both processes result in pro-asthmatic-like changes in ASM responsiveness. Additionally, siRNA directed against PDE4D5 selectively inhibits β2Rr-induced transcriptional upregulation of PDE4D5 in human ASM and this tool has been used to show that PDE4D5 activity is the crucial controlling factor that governs the magnitude, timing, and spatial characteristics of cAMP gradients produced in response to β2AR activation of human airways (Billington et al. 2008). With this in mind, disruption of the PDE4D5–β-arrestin complex may offer a novel therapeutic route to combat the onset of tolerance in response to chronic use of β2-adrenoceptor agonists (Fig. 3).

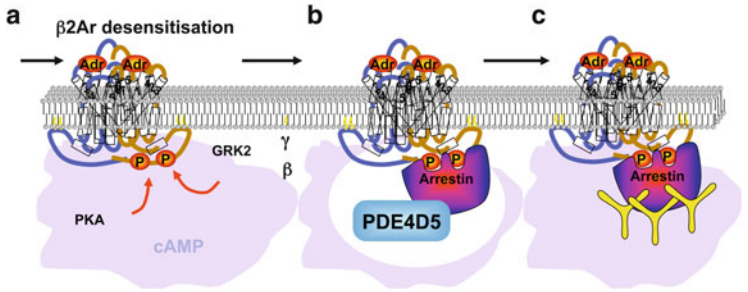

Fig. 3 PDE4D5 is recruited by β-arrestin to initiate desensitization of the β2-adrenergic receptor. (**a**) GRK2 and PKA phosphorylate sites on the β2AR to allow it to recruit β-arrestin. (**b**) β-arrestin sterically hinders the signal between Gs and the receptor while PDE4D5 hydrolyzes the second messenger cAMP (lilac). (**c**) Peptide disruptors of the β-arrestin–PDE4D5 association (*yellow Y-shape*) prevent hydrolysis of cAMP caused by overexpression of PDE4D5

5 Involvement of the PDE4–β-Arrestin Complex in Immune Cell Response

T-cell responses triggered by TCRs (T-cell receptors) originate from lipid rafts. Such structures can be considered as microdomains within the cell membrane that are enriched in cholesterol and sphingolipids. These domains act as signaling platforms that include or recruit protein complexes involved in T-cell response (Xavier et al. 1998). One mechanism by which signals are transduced following TCR simulation, is via the second messenger, cAMP. It has been established that stimulation of the TCR results in an elevation of cAMP (Ledbetter et al. 1986) that inhibits T-cell function and growth (Skalhegg et al. 1992) via a PKA-regulated signaling axis (Vang et al. 2001). Consequently, tight regulation of cAMP in the vicinity of the TCR is required for effective proximal T-cell signaling. As it was known that spatial and temporal control of cAMP gradients following activation of the β2AR was underpinned by a complex involving β-arrestin/PDE4 (Perry et al. 2002) and that PDE4 localizes to the TCR during activation (Arp et al. 2003), work was undertaken to determine if a similar β-arrestin-mediated desensitization mechanism occurred at the TCR. Surprisingly, it was shown that increases in lipid raft-associated PDE4 activity following simultaneous TCR and CD28 stimulation were a result of β-arrestin traslocation to these microdomains (Abrahamsen et al. 2004). The PDE4 pool recruited by β-arrestin was sufficient to suppress the inhibitory cAMP signal and effectively downregulate localized PKA activity leading to full T-cell response (Bjorgo and Tasken 2006). Further work added a new level of regulation to this signaling pathway by showing that the PDE4–β-arrestin complex also contained PKB and that this trimeric complex could associate with phosphatidylinositol-(3,4,5)-triphosphate (PIP3) (Bjorgo et al. 2011). Production of PIP3 at the membrane following TCR activation caused attraction of the PKB pleckstrin homology domain leading to recruitment of the PDE4–β-arrestin–PKB complex and subsequent degradation of the TCR-induced cAMP (Bjorgo et al. 2010).

6 PDE4–β-Arrestin Complex in the Desensitization of GPCRs in the Brain

It has been established that the signal termination mechanism of many of the GPCRs expressed in the brain involves homologous desensitization by β-arrestin [reviewed in Gainetdinov et al. (2004)]. Interestingly, most mu-opioid receptors (but not activated by morphine) recruit β-arrestin2 to block G-protein stimulation, arrest downstream signaling pathways, and enhance receptor internalization. The lack of morphine-induced β-arrestin2 recruitment is confounded by the fact that the acute analgesic effect of morphine is enhanced in β-arrestin2 knockout mice (Bohn et al. 2003). Further investigation has described a mechanism that is independent of mu-opioid receptor-driven β-arrestin2 recruitment but instead focuses on the lack of constitutive recruitment of PDE4 activity (by β-arrestin2) and increased cAMP levels in presynaptic locations within neurons (Bradaia et al. 2005). In β-arrestin2 knockout mice, enhanced PKA activity triggered by the rise in cAMP evoked a higher frequency of miniature inhibitory currents that resulted in an increased chance of neurotransmitter release downstream of calcium entry. This action conferred an enhanced ability of morphine to inhibit GABA release and highlights the importance of PDE4 recruitment by in β-arrestin2 in neuronal signaling.

A possible role for the β-arrestin–PDE4 complex has also been identified in the formation of associative fear memory. Following on from the observations that perfusion of PKA inhibitors into the lateral nucleus of the amygdala before conditioning resulted in the attenuation of fear memory (Schafe et al. 1999) and that PDE4D knockout mice also exhibit impaired fear conditioning (Rutten et al. 2008), a study was undertaken to ascertain whether β-arrestin had a role in this process. β-arrestin2 knockout mice had impaired conditioned fear memory that could be rescued by the overexpression of β-arrestin2 but not by a β-arrestin2 mutant that was defective in its ability to form a complex with PDE4 (Li et al. 2009b). Moreover, fear conditioning was shown to induce translocation of the β-arrestin–PDE4 complex to the membrane to attenuate inhibitory PKA signaling. Taken together, these data suggest that β-arrestin-mediated dynamic translocation of PDE4 activity is critical for the long-term potentiation at the lateral amygdalar synapses and for formation of conditioned fear memory (Li et al. 2009b).

From all of the work described above, it is clear that the dynamic repositioning of active PDE4 enzymes by β-arrestin is a pivotal part of the desensitization mechanism that acts to interdict the signals provided by many different kinds of activated GPCRs. All of these receptors use cAMP as a second messenger; therefore, it is apt that PDE4 (a cAMP-specific PDE) is the only member of PDE family that has been reported to complex with β-arrestin. Other systems, which use cGMP as an intracellular signaling molecule, have not, so far, been shown to use β-arrestin-mediated recruitment of cGMP-specific or dual cAMP-cGMP PDEs as a way to suppress effects of cyclic nucleotide synthesis. This may, however, change as a recent study has demonstrated that β-arrestin2 can co-localize with PDE1C in the olfactory epithelia of rodents (Menco 2005).

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Arrestins in Apoptosis

Seunghyi Kook, Vsevolod V. Gurevich, and Eugenia V. Gurevich

Contents

Abstract Programmed cell death (apoptosis) is a coordinated set of events eventually leading to the massive activation of specialized proteases (caspases) that cleave numerous substrates, orchestrating fairly uniform biochemical changes than culminate in cellular suicide. Apoptosis can be triggered by a variety of stimuli, from external signals or growth factor withdrawal to intracellular conditions, such as DNA damage or ER stress. Arrestins regulate many signaling cascades involved in life-or-death decisions in the cell, so it is hardly surprising that numerous reports document the effects of ubiquitous nonvisual arrestins on apoptosis under various conditions. Although these findings hardly constitute a coherent picture, with the same arrestin subtypes, sometimes via the same signaling pathways, reported to promote or inhibit cell death, this might reflect real differences in pro- and antiapoptotic signaling in different cells under a variety of conditions. Recent finding suggests that one of the nonvisual subtypes, arrestin-2, is specifically cleaved by caspases. Generated fragment actively participates in the core mechanism of apoptosis: it assists another product of caspase activity, tBID, in releasing

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cytochrome C from mitochondria. This is the point of no return in committing vertebrate cells to death, and the aspartate where caspases cleave arrestin-2 is evolutionary conserved in vertebrate, but not in invertebrate arrestins. In contrast to wild-type arrestin-2, its caspase-resistant mutant does not facilitate cell death.

Keywords Arrestin • Cell death • Apoptosis • Cell signaling • Cytochrome C • Caspases • JNKs

1 Apoptotic Pathways

Apoptosis is a form of programmed cell death (Vaux et al. 1994; Steller 1995) involving the activation of caspases (Thornberry and Lazebnik 1998; Crawford and Wells 2011). Caspases concentrate on key pathways, producing stereotypic morphological and biochemical changes in apoptotic cells (Dix et al. 2008; Mahrus et al. 2008; Chipuk et al. 2010). Apoptosis can be triggered by a number of factors including signaling via death receptors (DR); DNA damage by UV, γ-irradiation, or genotoxic drugs; load on endoplasmic reticulum (ER); withdrawal of growth or trophic factors; oxidative stress; and a large number of other factors.

The apoptotic pathway initiated by DR activation is known as extrinsic pathway (Fig. 1). Eight members of the DR family have been described, including the best-studied tumor necrosis factor receptor alpha 1 (TNFR1), Fas (also known as CD95), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1), and TRAILR2 (Lavrik et al. 2005). The activation of DR by corresponding ligands leads to the formation of signaling complexes assembled at the intracellular surface of the receptor. Fas and TRAILR1/2 recruit what is known as death-inducing signaling complexes (DISC) containing FADD (Fas-Associated Death Domain), pro-caspase-8, and the long and short forms of the cellular FLICE inhibitory protein ($FLIP_{L/S}$) as main components (Lavrik et al. 2005; Guicciardi and Gores 2009). The formation and internalization of DISC result in the activation of initiator caspase-8 that cleaves and activates effector caspase-3, caspase-6, and caspase-7, triggering apoptotic cell death (Lavrik et al. 2005; Guicciardi and Gores 2009). In cell type I, DISC is effectively internalized, resulting in massive caspase-8 activation sufficient to activate downstream caspases (Scaffidi et al. 1998, 1999; Fulda et al. 2001). In cell type II, lower level of DISC formation results in weak caspase-8 activation that requires amplification to trigger apoptosis. The amplification cascade includes capspase-8-mediated cleavage of the member of the BCL-2 family BID to yield truncated BID (tBID) that translocates to the mitochondria inducing the cytochrome C release (Li et al. 1998; Luo et al. 1998) via a still poorly understood mechanism (Chipuk and Green 2008). The cytochrome C release results in the formation of the apoptosome and the activation of caspase-9, which in turn cleaves and activates the effector caspases (Danial and Korsmeyer 2004; Bratton and Salvesen 2010).

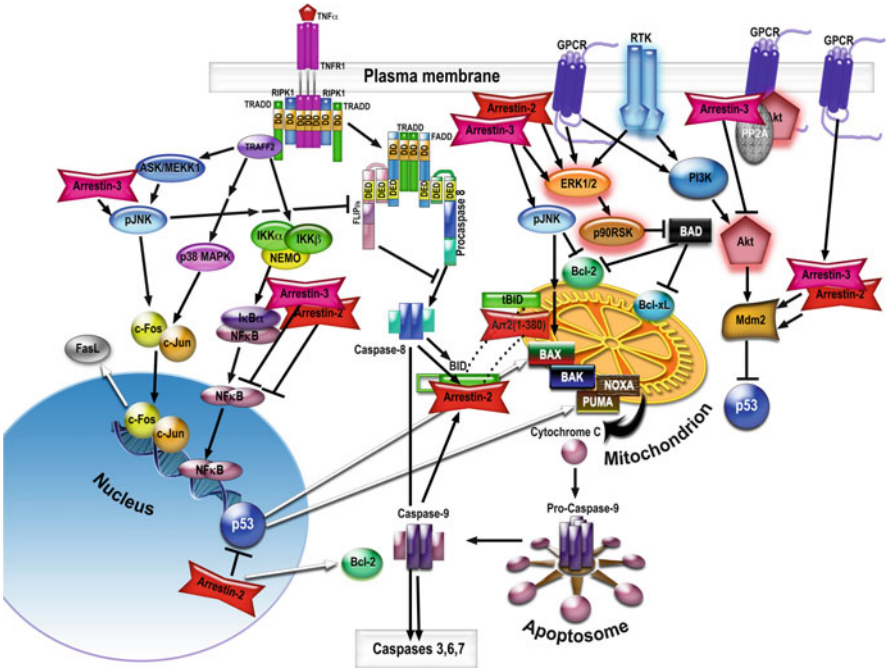

Fig. 1 Numerous arrestin functions play a role in apoptosis and cell survival. The activation of the death receptor TNFR1 by TNFα results in the assembly of the multi-protein complex I that activates the NFκB pathway along with JNK and p38 pathways. The NFκB signaling is antiapoptotic, mostly via transcriptional activation of FLIP that inhibits caspase-8 activation. Activation of JNK and p38 promotes apoptosis via transcriptional as well as posttranscriptional mechanisms (see text). Signaling complex II formed following internalization and reshuffling of signaling proteins directly activates caspase-8 that cleaves and activates effector caspase-3, caspase-6, and caspase-7 initiating apoptosis. Apoptotic pathway induced via death receptor is known as the extrinsic pathway. Caspase-8 also cleaves BID, generating tBID that translocates to the mitochondria inducing BAX-BAK oligomerization and cytochrome C release. Cytochrome C organizes apoptosome, activating caspase-9, which then activates massive amounts of caspase-3. Apoptosis can also be initiated by a variety of stress stimuli that engage the mitochondria-based apoptotic pathway termed the intrinsic pathway. Specifically, genotoxic drugs and other stimuli causing DNA damage initiate the intrinsic pathway via transcription factor p53 that upregulates pro-apoptotic genes BAX, PUMA, and NOXA. Arrestins have been shown to promote activation of MAP kinases by scaffolding (Chapters 12–14). GPCR-dependent activation of ERK1/2 by both nonvisual arrestins has been shown to provide protection against apoptosis induced by various agents in many cell types. Arrestin-3, but not arrestin-2, is able to activate neuron-specific JNK3 isoform. Although JNK3 has been shown to play an important role in neuronal apoptosis, the evidence of the role of arrestin-3-dependent JNK3 activation in neuronal death is so far lacking. Both arrestin isoforms interact with IκBα, an inhibitory protein that binds NFκB and holds it in the cytosol, preventing NFκB-dependent antiapoptotic transcription. Arrestins have been demonstrated to regulate the activity of the pro-survival Akt pathway. Arrestin-3 is able to reduce the Akt activity via scaffolding Akt together with PP2A that dephosphorylates Akt in response to D2 dopamine receptor stimulation. Arrestins have been shown to stabilize Mdm2 and promote its activation, as well as affect p53 degradation and level (see text for details). Arrestin-2 also interacts with p53 in the nucleus, acting as important adaptor for Mdm2 required for Mdm2-dependent p53 degradation. Interestingly, caspase-8 that cleaves BID also cleaves arrestin-2 at the C-terminus generating Arr2-(1-380) fragment. Arr2-(1-380) translocates to the mitochondria,

In type II cells, Fas-induced apoptosis could be blocked by antiapoptotic BCL family members such as BCL-2 and BCL-XL (Scaffidi et al. 1998, 1999; Fulda et al. 2001). Therefore, tBID serves as a mediator of the positive feedback, or amplification, loop involving the mitochondria-dependent apoptotic signaling.

Stimulation of TNFR1 and similar DR results in the formation of two signaling complexes. Complex I assembled at the membrane includes TRADD (TNFR-associated death domain protein), RIPK1 (receptor-interacting serine/threonine-protein kinase 1), and TRAF2/5 (TNFR-associated factor) as main components (Micheau and Tschopp 2003; Lavrik et al. 2005). Complex I mediates TNFR1-induced activation of the NFκB and JNK pathways (Dempsey et al. 2003; Lavrik et al. 2005). The NFκB pathway is activated via recruitment of the IKK complex in the TRADD-dependent manner with participation of RIPK1 and TRAF2/5/6 through a series of K63 "nondestructive" ubiquitination events (Micheau and Tschopp 2003; Ea et al. 2006; O'Donnell and Ting 2010; Pobezinskaya and Liu 2012). Recruitment of the IKK complex leads to the phosphorylation of the NFκB inhibitory protein IκBα, with its subsequent degradation, and activation of NFκB-dependent transcription of antiapoptotic genes such as cFLIP, cIAP1, cIAP2, BCL-XL, and XIAP (Kreuz et al. 2001; Micheau et al. 2001; Dempsey et al. 2003; Chipuk et al. 2010). JNK activation by TNFR1 is TRAF2 dependent (Natoli et al. 1997; Reinhard et al. 1997; Yuasa et al. 1998; Habelhah et al. 2004). TNFR1 also activates the p38 pathway in a TRAF2- and RIPK1-dependent manner (Yuasa et al. 1998; Lee et al. 2003). The MAP kinase pathways are activated via recruitment and activation of upstream kinases MEKK1, ASK1, or TPL2 to TRAF2 (Nishitoh et al. 1998; Yuasa et al. 1998; Das et al. 2005) (see also chapters "Arrestin-Dependent Activation of ERK and Src Family Kinases," "Arrestin-Dependent Activation of JNK Family Kinases," and "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences").

Complex I is internalized and transformed in the cytosol into complex II by exchange of signaling proteins associated with TNFR1. FADD and pro-caspase-8 are recruited, leading to caspase-8 activation and initiation of apoptosis (Micheau and Tschopp 2003; Schneider-Brachert et al. 2004). Unlike Fas and TRAIL receptors, TNFR1 is mostly involved in mediating inflammation and not cell death, and the outcome of the TNFR1 stimulation is cell type dependent. Inhibition of RNA or protein synthesis resulting in the blockade of complex I-mediated pro-survival NFκB-mediated signaling is required to induce apoptosis via TNFR1 stimulation

Fig. 1 (continued) directly binds tBID, and greatly enhances its ability to induce cytochrome C release from mitochondria, thereby promoting apoptosis. *Black arrows* indicate direct or indirect posttranslational activation; *black bar*—inhibitory modification; *white arrows*—transcriptional upregulation; *dotted line*—translocation. *Abbreviations*: *TNFR1* TNFα receptor 1, *RIPK1* receptor-interacting serine/threonine-protein kinase 1, *FADD* Fas-associated death domain protein, *TRADD* TNF receptor-associated death domain (TRADD), *TRAF* TNF receptor-associated factor, *FLIP* FLICE-like inhibitory protein (a.k.a. CFLAR, CASP8, and FADD-like apoptosis regulator), *DD* death domain, *DED* death effector domain, *RTK* receptor tyrosine kinase, *GPCR* G protein-coupled receptor

in most cell types. Blockade of NFκB signaling promotes TNFR1-induced apoptosis mostly by blocking the synthesis of cFLIP that inhibits caspase-8 activation (Kreuz et al. 2001; Micheau et al. 2001). Alternatively, TNFR1 signaling could be switched from pro-survival to pro-apoptotic mode by Smac, also known as Diablo (or its mimetics). Smac is a protein released from the mitochondria together with cytochrome C that interacts with and inhibits apoptotic inhibitors XIAP, cIAP1, and cIAP2 (Chai et al. 2000; Du et al. 2000). Smac can also trigger RIPK1-dependent mode of capsase-8 activation by promoting degeneration of IAPs (Wang et al. 2008). The positive regulation of TNFR1 apoptotic signaling by Smac/Diablo released from the mitochondria is another mitochondria-based amplification pro-apoptotic mechanism.

The apoptotic pathway mediated by the release of pro-apoptotic factors from the mitochondria followed by the formation of apoptosome, activation of initiator caspase-9, and subsequent activation of effector caspases is referred to as the intrinsic pathway (Danial and Korsmeyer 2004) (Fig. 1). The intrinsic apoptotic pathway is triggered by a large variety of stimuli including DNA damage, withdrawal of growth factors, hypoxia, or endoplasmic reticulum stress. The signaling converges on the mitochondria where the interplay of pro- and anti-apoptotic BCL family members regulates cytochrome C release, although the exact biochemical mechanism of this process has not been elucidated (Danial and Korsmeyer 2004; Youle and Strasser 2008). Effectors BAK and BAX oligomerize and form pores in the outer mitochondrial membrane (Wei et al. 2000), allowing cytochrome C (and other mitochondrial proteins such as Smac/Diablo) to escape to the cytoplasm (Lindsten et al. 2000; Wei et al. 2001). The biochemical nature of this pore and the number of BAK or BAX proteins necessary to create it remain unknown (Youle and Strasser 2008). BCL-2 homology 3 (BH3)-only members of the BCL-2 family either directly activate BAX and BAK and induce cytochrome C release or do so indirectly via antagonistic interaction with antiapoptotic members of the same family. It is still unclear how exactly the interactions of pro- or anti-apoptotic BCL-2 proteins with BAK and BAX affect the pore formation process (Chipuk and Green 2008; Youle and Strasser 2008). Truncated BID (tBID) cleaved by caspase-8 activated in the extrinsic apoptotic pathway is the most potent cytochrome C releaser among BH3 proteins (Korsmeyer et al. 2000; Wei et al. 2000; Lovell et al. 2008), providing a strong amplification signal for apoptosis induced by DR activation. The release of cytochrome C from mitochondria promotes the formation of a structure known as apoptosome composed of cytochrome C, pro-caspase-9, and apoptotic protease activation factor 1 (Apaf 1) that results in capsape-9 activation (Danial and Korsmeyer 2004; Bratton and Salvesen 2010). Active caspase-9 cleaves and activates effector caspases. Thus, the apoptosome serves a function analogous to that of DISC, i.e., activation of an initiator caspase, albeit achieved via a different molecular mechanism. The apoptosome formation that promotes massive activation of executioner caspase-3/caspase-6/caspase-7 is the key checkpoint in cell commitment to death (Youle and Strasser 2008). Stressful stimuli capable of engaging the intrinsic apoptotic pathway do so by increasing the expression of pro-apoptotic proteins or via direct inactivation of antiapoptotic proteins. Thus,

DNA damage caused by UV, γ-irradiation, or genotoxic drugs results in stabilization of the transcription factor p53 that translocates to the nucleus promoting the expression of multiple pro-apoptotic genes and subsequent cell death (Oda et al. 2000; Nakano and Vousden 2001; Yu et al. 2001; Villunger et al. 2003; Naik et al. 2007; Michalak et al. 2008). Growth factors such as NGF and BDNF can induce neuronal death acting via p75 receptor, which is a member of the death receptor superfamily that includes Fas and TNFR1 (Chao 1994; Sessler et al. 2013). The mechanisms of this apoptosis are poorly understood but appear to involve JNK activation and downstream engagement of the mitochondrial pathway (Harrington et al. 2002; Salehi et al. 2002; Nykjaer et al. 2005; Ichim et al. 2012; Sessler et al. 2013). Some trophic factor receptors seem to function as so-called dependence receptors that induce positive apoptotic signaling in the absence of ligands. The survival of certain types of cells in culture is strictly dependent on the presence of specific trophic factors, and apoptosis of many cultured cell lines can be induced by serum withdrawal. Apoptosis mediated by dependence receptors involves caspase interaction and caspase-mediated cleavage of the receptor cytoplasmic domain yielding a pro-apoptotic peptide that mediates downstream signaling including transcriptional activation of pro-apoptotic genes (Rabizadeh and Bredesen 2003; Bredesen et al. 2005; Goldschneider and Mehlen 2010; Ichim et al. 2012). Thus, multiple cellular signaling pathways impact the core apoptotic machinery, thereby affecting cell death and survival.

2 Signaling Mechanisms in Apoptosis

Given the irreversible nature of apoptosis, it is not surprising that large number of checks and balances is incorporated into the core apoptotic mechanisms. Furthermore, multiple cellular signaling pathways impact the function of most proteins involved in apoptosis. A good example is TNFR1: its activation triggers anti- and pro-apoptotic mechanisms, such as activation of the NFκB and JNK pathways. The NFκB pathway suppresses apoptosis via transcriptional upregulation of antiapoptotic genes (Dempsey et al. 2003; Lavrik et al. 2005; Chipuk et al. 2010). The activity of the NFκB pathway is regulated by inhibitory protein IκBα that keeps NFκB inactive in the cytoplasm. IκBα is phosphorylated by the IKK complex, which induces its polyubiquitination and proteasomal degradation (Chen et al. 1996; Roff et al. 1996; Napetschnig and Wu 2013). NFκB, thus released, translocates to the nucleus and activates transcription (Napetschnig and Wu 2013). The IKK complex is composed of two related catalytic subunits, IKKα and IKKβ, and an important although catalytically inactive component NEMO/IKKγ (Chen et al. 1996; DiDonato et al. 1997; Mercurio et al. 1997; Yamaoka et al. 1998). IKKβ phosphorylation is required for the NFκB activation via so-called canonical pathway (turned on by TNFα), and it appears that TAK1, which also serves as an MAPKKK in the JNK pathway, can phosphorylate IKKβ in the activation loop (Ninomiya-Tsuji et al. 1999; Wang et al. 2001; Sato et al. 2005). MEKK3, another

upstream MAP kinase, has also been implicated in TNFα-induced IKK activation (Yang et al. 2001). NEMO specifically binds to linear and K63 polyubiquitin chains, which is critical for the activation of the TNFα-induced IKK recruitment and NFκB activation (see Napetschnig and Wu (2013) and references therein). The IKK complex could be activated by receptor belonging to Toll-like-interleukin-1 receptor superfamily involved in the innate immunity responses via recruitment of TRAF6 (Bradley and Pober 2001).

2.1 The JNK Pathway

Active NFκB leads to a rapid quenching of TNFR1-induced JNK activation. The proposed mechanisms of NFκB-induced suppression of JNK activity include upregulation of Gadd45 beta factor that inhibits MKK7, an upstream kinase activating JNK (De Smaele et al. 2001; Papa et al. 2004), and upregulation of XIAP (Tang et al. 2001). However, cells lacking Gadd45 beta or XIAP showed TNFR1-induced JNK activation similar to that of wild type (Amanullah et al. 2003; Kucharczak et al. 2003). An alternative mechanism involves TNFα-generated reactive oxygen species that inhibit JNK phosphatases (Kamata et al. 2005), which normally ensure low level of JNK activity (Cavigelli et al. 1996). Several studies reported that sustained JNK activation augments TNFR1-induced death in cells with deficient NFκB pathway (De Smaele et al. 2001; Tang et al. 2001). Therefore, JNK activity could play a decisive role in the outcome of the TNFR1 activation if the function of the NFκB pathway is compromised due to genetic defects or drug action. JNK activation plays the key role in apoptosis induced by UV irradiation and genotoxic drugs mediated by the intrinsic apoptotic pathway (Zanke et al. 1996; Tournier et al. 2000). JNK3, a JNK isoform selectively expressed in neurons, has been shown to be involved in apoptosis caused by excitotoxic (Yang et al. 1997) or other toxic (Namgung and Xia 2000) agents and by growth factor deprivation (Bruckner et al. 2001; Eilers et al. 2001; Coffey et al. 2002; Barone et al. 2008; Ambacher et al. 2012). JNK activation plays an important role in neuronal apoptosis following focal ischemia (Okuno et al. 2004; Gao et al. 2005) and in beta-amyloid-induced neuronal apoptosis (Morishima et al. 2001; Yao et al. 2005).

It appears that sustained JNK activation is required to promote apoptosis, whereas transient JNK activity is involved in cell proliferation and survival (Sánchez-Perez et al. 1998; Chen and Tan 2000; Dhanasekaran and Reddy 2008). The pro-apoptotic action of JNK is in many cases transcriptional, mediated by JNK-dependent phosphorylation and transactivation of the transcription factor c-jun and subsequent expression of pro-apoptotic genes (Behrens et al. 1999; Coffey et al. 2002; Barone et al. 2008; Dhanasekaran and Reddy 2008). Ironically, the nature of genes induced by JNK activation has never been extensively defined. One gene proposed to be transcriptionally activated by JNK and involved in

apoptosis was Fas ligand (Le-Niculescu et al. 1999; Mansouri et al. 2003; Wang et al. 2004). Sustained JNK activation may promote TNFα-induced apoptosis via JNK-mediated activation of E3 ubiquitin ligase Itch that ubiquitinates cFLIP, leading to its proteasomal degradation and, subsequently, enhanced caspase-8 activation (Chang et al. 2006). JNK activation can also lead to caspase-8-independent cleavage of BID at a different site, and the cleaved product, jBId, translocated to the mitochondria, inducing preferential release of Smac/Diablo; this, in its turn, promotes TNFα-dependent apoptosis by disrupting the TRAF2-cIAP1 interaction inhibitory for caspase-8 activation (Deng et al. 2003). The JNK activity has been shown to affect the p53-dependent apoptosis in different cell types via p53 phosphorylation that alters the activity or stability (Fogarty et al. 2003; Oleinik et al. 2007). JNK is also known to phosphorylate members of the BCL-2 family, thus directly affecting their function (Yamamoto et al. 1999; Donovan et al. 2002; Lei and Davis 2003; Putcha et al. 2003; Okuno et al. 2004). JNK can also alter their functions indirectly by phosphorylating interacting proteins. The best known such effect is translocation of BAX to the mitochondria promoted by JNK-dependent phosphorylation of BAX cytoplasmic anchoring protein 14-3-3 (Tsuruta et al. 2004; Gao et al. 2005). The function of neuron-specific JNK3 isoform in neuronal apoptosis caused by ischemia/hypoxia is believed to be mediated by induction of BIM and other pro-apoptotic genes (Kuan et al. 2003; Zhang et al. 2006; Zhao et al. 2007).

2.2 The p53 Pathway

The tumor suppressor protein p53 is a transcription factor that mediates apoptosis caused by multiple stressors, including DNA damaging agents such as UV, γ-irradiation, or genotoxic drugs (e.g., topoisomerase inhibitor etoposide) (Vousden and Lane 2007; Delbridge et al. 2012). As mentioned above, p53 promotes cell death by increasing the expression of pro-apoptotic genes such as BAX, PUMA, and NOXA (Oda et al. 2000; Nakano and Vousden 2001; Yu et al. 2001), with PUMA and, to a lesser degree, NOXA being the main culprits (Villunger et al. 2003; Naik et al. 2007; Michalak et al. 2008). The level of p53 in cells is tightly controlled to keep the balance between cell death and tumor development that occurs when p53 function is compromised (Delbridge et al. 2012). Oncoprotein RING finger E3 ubiquitin ligase Mdm2 is the main negative regulator of p53 that ubiquitinates p53, promoting its proteasomal degradation (Fang et al. 2000; Honda and Yasuda 2000). Apparently, Mdm2 requires collaboration with MdmX, a related protein without intrinsic E3 ligase activity, to polyubiquitinate p53 (Parant et al. 2001; Wang et al. 2011). In its turn, p53 stimulates Mdm2 transcription. Thus, Mdm2 and p53 form a regulatory feedback loop that is strongly impacted by cellular stress, resulting in inactivation of Mdm2 and activation of p53 (Stommel and Wahl 2005). The key importance of p53 for survival and Mdm2 for its regulation is strongly supported by the fact that mice lacking Mdm2 die in early

embryogenesis, whereas mice lacking both Mdm2 and p53 are grossly normal (Jones et al. 1995; Montes de Oca Luna et al. 1995). Recent data demonstrate that, in addition to its transcriptional role, p53 regulates the mitochondrial apoptotic pathway in a transcription-independent manner via direct interaction with BCL-2 proteins at the mitochondria (Chipuk et al. 2003, 2004, 2005).

3 Arrestins Regulate Apoptosis via Signaling Mechanisms

The canonical mode of arrestin function in homologous desensitization of GPCRs involves arrestin binding to phosphorylated activated receptors that terminates G protein activation by blocking its access to the receptor cytoplasmic surface (Wilden 1995; Krupnick et al. 1997). Ubiquitous arrestin-2 and arrestin-3[1] regulate most GPCRs, suppressing G protein activation (Attramadal et al. 1992; Lohse et al. 1992). Arrestins also bind numerous non-receptor partners, thus regulating multiple cellular signaling pathways (Lefkowitz and Shenoy 2005; Gurevich and Gurevich 2006). Since many of these pathways are involved in "life-or-death" decisions in the cell, arrestins have been reported to influence cell death and survival via signaling mechanisms (Fig. 1). Indeed, considering a wide variety of signaling pathways regulated by arrestins (Gurevich and Gurevich 2006; Luttrell and Miller 2013), it would have been surprising if arrestins did not affect apoptosis. However, the data on the exact mechanisms involved are remarkably fragmentary.

GPCR stimulation followed by G protein activation can induce pro-apoptotic signaling, and arrestins would counteract that signaling simply by virtue of desensitizing the offending receptors. One example of such situation is apoptosis induced by stimulation of various GPCRs in arrestin-2/arrestin-3 DKO MEFs, with expression of either arrestin protecting these cells (Revankar et al. 2004). The molecular mechanism of GPCR-induced apoptosis, which occurs via the intrinsic pathway, includes the activation of p38, JNK, phosphatidylinositol 3-kinase (PI3K), and Gi/o-dependent signaling. Interestingly, excessive signaling due to defective receptor desensitization in the absence of arrestins does not seem to be the culprit. At least in case of the N-formyl peptide receptor, which is internalized in arrestin-independent manner but requires arrestins for recycling (Vines et al. 2003), receptor phosphorylation and internalization were required to induce apoptosis. Arrestin interaction with adaptor protein-2 (AP-2) participating in post-endocytotic receptor trafficking (see chapter "β-Arrestins and G Protein-Coupled Receptor Trafficking") was involved in the protection from GPCR-induced apoptosis by arrestins (Wagener et al. 2009).

[1] Different systems of arrestin names are used in the field and in this book. We use systematic names of arrestin proteins: arrestin-1 (historic names S-antigen, 48-kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin-1), arrestin-3 (β-arrestin-2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called "*arrestin-3*" in the HUGO database).

In an alternative case scenario, arrestins could be involved in apoptosis-related signaling by various receptors, and as recent studies indicate, their role is not limited to GPCRs. Whether the outcome of arrestin-dependent signaling is pro-survival or pro-apoptotic depends on the specific configuration of the signaling system in which they act. When arrestin-mediated signaling results in the activation of pro-survival pathways such as ERK or Akt, arrestins provide cytoprotection. In the opposite case, when the arrestin action leads to the suppression of pro-survival or induction of pro-apoptotic signaling, arrestins serve to facilitate cell death. Thus, arrestin-2 protected cells from apoptosis caused by serum deprivation via NK1 receptor and arrestin-dependent ERK1/2 activation (DeFea et al. 2000). Similarly, arrestin-2-dependent ERK activation mediated protective effect of glutamate acting via metabotropic glutamate receptor 1 (mGluR1) against serum-deprivation-induced apoptosis (Emery et al. 2010). Both arrestin-2 and arrestin-3 mediate transactivation of the epidermal growth factor (EGF) receptor by the Gq-coupled receptor of neuropeptide urotensin II (Esposito et al. 2011). Urotensin II, which is expressed in the nervous, cardiovascular, and urogenital systems, and its receptor are upregulated in the pathological heart (Zhu et al. 2006), and this increase seems to be protective, since treatment with urotensin II antagonist exacerbates heart pathology and promotes apoptosis of cardiomyocytes, the effect linked to reduced EGF receptor transactivation and resulting ERK activity (Esposito et al. 2011). Arrestin-3 mediated the protection conferred by the angiotensin II receptor 1A to primary rat vascular smooth muscle or to HEK293 cells against hydrogen peroxide (H_2O_2)- or etoposide-induced apoptosis (Ahn et al. 2009). Arrestin-3-dependent ERK activation followed by activation of the P90 ribosomal S6 kinase (P90RSK) and activation of Akt was required for its antiapoptotic activity. P90RSK and Akt in their turn phosphorylated pro-apoptotic BCL-2 protein Bad at Ser^{112} and Ser^{136}, respectively, thus inhibiting its pro-apoptotic activity. A similar mechanism was described for the arrestin-2 role in the protection against glucose deprivation-induced apoptosis afforded by stimulation of the glucagon-like peptide-1 Gs-coupled receptor (GLP-1) to pancreatic beta cells (Quoyer et al. 2010). Arrestin-2-mediated ERK activation resulted in the activation of the P90RSK, leading to phosphorylation of Bad at Ser^{112}. Apparently, in some cases arrestin-dependent ERK activation could be harmful to cells. Thus, dopamine at high concentration acting at the D1 dopamine receptor has been reported to cause apoptotic death of primary and cultured neuronal cells via sustained arrestin-2-dependent ERK activation in the cytosol (Chen et al. 2004, 2009).

Arrestins are known to be involved in the regulation of the pro-survival Akt pathway. Previously, arrestin-3 (and to a lesser extent arrestin-2) has been shown to reduce the activity of the Akt pathway by scaffolding Akt with protein phosphatase 2 at the D2 dopamine receptor, which resulted in dephosphorylation of Akt at its main activating residue Thr^{408} (Manning and Cantley 2007). It has not been examined whether this regulatory effect of arrestin-3 on the Akt pathway activity plays any role in apoptosis, which appears likely. Arrestin-2 is protecting from serum deprivation-induced apoptosis by coupling the insulin-like growth factor 1 receptor to the activation of PI3K and subsequent activation of the Akt pathway

(Povsic et al. 2003). This signaling process occurs independently of the tyrosine kinase activity of the receptor, Gi, or ERK activity. Platelet-activating factor acting at its receptor induces apoptosis in colon cancer cells by promoting dephosphorylation of Akt at Ser^{473} via assembly at the receptor of arrestin-2 and PH domain and leucine-rich repeat protein phosphatase 2 that dephosphorylates Akt at this residue (Crotty et al. 2013; Xu et al. 2013). This is a novel mode of arrestin-dependent inhibition of the Akt pathway that may play a role in apoptosis via suppression of the pro-survival Akt signaling. The glycogen synthase kinase-3 (GSK3) is the main substrate of Akt. This kinase is constitutively active, and it is inhibited by Akt phosphorylation (Manning and Cantley 2007). GSK3β isoform is known to potentiate mitochondrial apoptotic signaling (Hetman et al. 2000; Beurel and Jope 2006; Eom et al. 2007; Mishra et al. 2007; Watcharasit et al. 2008). The pro-survival effect of the PI3K/Akt activation is largely mediated by inhibition of GSK3β. Arrestins, particularly arrestin-3, by modulating the activity of PI3K and/or Akt, could impact the GSK3β-dependent apoptosis. This notion is supported by the fact that MEFs lacking arrestin-3 demonstrate higher level of GSK3β activity (lower phosphorylation) coupled with increased apoptotic death (Li et al. 2010).

Arrestins turned out to be involved in the regulation of the pro-survival NFκB pathway. Arrestin-3 has been reported to inhibit NFκB activation induced by TNFα (Gao et al. 2004). The mechanism of the inhibition involves direct interaction of arrestin-3 with the inhibitor of NFκB IκBα, which prevents phosphorylation and degradation of the latter, thus precluding the activation of NFκB. In this study, arrestin-3 was shown to significantly inhibit TNFα-induced translocation of NFκB p65 subunit into the nucleus and transcription of NFκB-dependent genes. Importantly, arrestin-3 association with IκBα, as well as its effect on the NFκB activation, was significantly increased by stimulation of β2-adrenergic receptor (b2AR). Thus, arrestin-3 mediates the effect of b2AR on the NFκB activity, which may play a role in the sympathetic regulation of TNFα immune responses. In this study, the TNF-α-induced apoptosis was not directly examined, although arrestin-3-dependent inhibition of the pro-survival signaling induced by TNFR1 should be expected to favor cell death. Interestingly, arrestin-2 was reported to be unable to stabilize IκBα and affect the p65 translocation or the expression of NFκB target genes. Another group, however, demonstrated that both arrestin isoforms interacted with IκBα, significantly inhibiting the NFκB activity induced by various stimuli (Witherow et al. 2004). Moreover, knockdown of arrestin-2 and not arrestin-3 resulted in significant increase in the TNFα-induced activation of NFκB, suggesting that arrestin-2 isoform is the prime regulator of the NFκB activation in response to TNFα. The NFκB is also activated by UV irradiation, and arrestin-3 was shown to suppress that activation via interaction with IκBα facilitating apoptotic cell death (Luan et al. 2005). The ability of arrestin-3 to interact with IκBα was blocked by its phosphorylation by casein kinase II, and stimulation of b2AR in epidermal cells promoted arrestin-3 dephosphorylation together with arrestin-3-dependent suppression of NFκB activity. Therefore, arrestin-3 facilitated UV-induced apoptosis in the b2AR-dependent manner via inhibition of the NFκB pathway. An alternative mechanism of arrestin-dependent regulation of the NFκB activity was demonstrated

in the immune system. Arrestin-3 was shown to interact with TRAF6 and inhibit TRAF6 autoubiquitination and oligomerization after stimulation of interleukin receptors, leading to suppression of the NFκB activation and immunological response to endotoxin challenge (Wang et al. 2006). Since TRAF6 is also involved in the TNFR1-induced NFκB activation, suppression of this effect may also favor apoptosis instead of cell survival.

An alternative mechanism of arrestin contribution to apoptosis caused by DNA damage is through its modulation of the p53 pathway. Arrestin-3 has been shown to bind E3 ubiquitin ligase, Mdm2, but not MdmX (Wang et al. 2003b), the key regulator of p53-dependent apoptosis mediated by the intrinsic pathway. Arrestin-3 binding to Mdm2 inhibited Mdm2 self-ubiquitination and Mdm2-dependent p53 ubiquitination, thus suppressing p53 degradation and promoting apoptosis. It appears somewhat inconsistent that arrestin-3 stabilizes both Mdm2 and p53, although normally high level of Mdm2 leads to a reduction in p53, and Mdm2 needs to be destabilized to allow the p53 level to rise (Stommel and Wahl 2005; Vousden and Lane 2007). Importantly, arrestin-3 binding to Mdm2 was reported to be strongly promoted by the activation of GPCRs such as δ-opioid, bradykinin, or b2AR. In this situation, arrestin-3 acted as a pro-apoptotic agent facilitating DNA damage-induced apoptosis. Conversely, arrestin-2, but not arrestin-3, recruited to active b2AR has been reported to facilitate Akt-mediated activation of Mdm2 promoting Mdm2-dependent degradation of p53 (Hara et al. 2011). Reduced level of p53 leads to the accumulation of stress-induced DNA damage in cultured cells and in the thymus in vivo, presumably due to defective p53-dependent apoptosis of damaged cells. Similarly, behavioral restrained stress leads to a reduction in the p53 level and accumulation of DNA damage in the mouse frontal cortex in the b2AR- and arrestin-2-dependent manner (Hara et al. 2013). Thus, arrestin-2 appears to play the antiapoptotic role via its activation of Mdm2. It remains unclear whether the opposite functions ascribed to arrestins in these studies could be explained away by the difference in arrestin isoforms, arrestin-3 versus arrestin-2, acting via different mechanism, direct interaction with Mdm2 versus indirect activation by Akt-dependent phosphorylation. Importantly, in these studies the mode of apoptosis, DNA damage induced, was the same, and the stimulating factor such as b2AR activation was also similar.

Although the role of JNK pathway in apoptosis is reasonably well established, and arrestin-3 is known to activate JNK3 (McDonald et al. 2000; Miller et al. 2001; Song et al. 2009a; Seo et al. 2011; Zhan et al. 2011, 2013; Breitman et al. 2012), this function of arrestins in apoptosis received surprisingly little attention. This is possibly because JNK3 expression is largely limited to the nervous system, with lower levels in the heart and testes (Gupta et al. 1996; Martin et al. 1996). Both arrestin isoforms attenuated H_2O_2-induced apoptosis by suppressing the JNK activation via direct interaction with apoptosis signal-regulated kinase-1 (ASK1), the upstream kinase (MAPKKK) in the JNK pathway (Zhang et al. 2009). Arrestin-ASK1 interaction, which was increased by H_2O_2, promoted ASK1 ubiquitination, via recruitment of E3 ligase CHIP, and subsequent proteasomal degradation, resulting in reduced JNK activation and increased cell survival without apparent

contribution from receptors. Ironically, this is the opposite paradigm to the classic arrestin-mediated JNK3 activation based on scaffolding by arrestin-3 of JNK3 upstream kinases, leading to enhanced JNK3 activation (McDonald et al. 2000). In the study by Zhang et al., the authors claim that arrestin-3-dependent JNK3 activation facilitated neuronal apoptosis following ischemia (Zhang et al. 2012). However, no evidence of role of arrestin-3-dependent JNK3 activation was presented. Instead, the experiments demonstrated reduced JNK3 activation by angiotensin II type 1 receptor antagonist losartan accompanied by protection against ischemia/reperfusion-induced neuronal death.

Arrestin-3 has been shown to mediate endocytosis of the type III transforming in the serum starvation condition growth factor-beta (TGFβ) receptor (TGFβRIII) in complex with TGFβRII and to reduce TGF-beta signaling (Chen et al. 2003). The loss of arrestin-3 has been shown to increase the rate of TGFβ-induced apoptosis, but the effect was mediated by enhanced TGFβ-dependent activation of p38 MAP kinase and not by Smad activation (McLean et al. 2013). The arrestin-3 effect on the p38 activity is likely mediated by its effect on the trafficking of TGFβ receptors, since the surface expression of the receptors increases upon the loss of arrestin-3. Arrestin function in apoptosis could be mediated by interactions with proteins outside of canonical pro- or antiapoptotic pathways. For example, arrestin-2 has been shown to protect human urothelial cells from staurosporine-induced apoptosis in b2AR-dependent manner via interaction with 27-kDa heat shock protein (Rojanathammanee et al. 2009).

Arrestins mostly regulate apoptosis via signaling mechanisms in the cytosol, although both are known to shuttle between the cytosol in the nucleus (Scott et al. 2002; Wang et al. 2003a; Song et al. 2006). Arrestin-3, but not arrestin-2, possesses a strong nuclear export signal and is able to relocalize nuclear proteins such as JNK and Mdm2 from the nucleus to the cytosol (Scott et al. 2002; Wang et al. 2003b; Song et al. 2006). Arrestin-2, on the other hand, has a single amino acid difference with arrestin-3 in the corresponding region, and unless the nuclear export signal is engineered, it is unable to relocalize its binding partners from the nucleus (Wang et al. 2003a; Song et al. 2006). Arrestin-2 possesses nuclear localization signal (Hoeppner et al. 2012) and has been reported to localize to the nucleus in some cell types (Hoeppner et al. 2012) and perform nuclear functions (Kang et al. 2005). Therefore, arrestins, arrestin-2 in particular, could interfere with apoptosis via signaling in the nucleus. Indeed, arrestin-2 has been shown to confer cytoprotection by stimulating transcription of antiapoptotic BCL-2 and thus promoting the survival of CD4+ native and activated T cells (Shi et al. 2007). Nuclear arrestin-2 also interacts with p53, acting in a somewhat poorly defined role of E3 ligase "adaptor" required for Mdm2 to ubiquitinate p53 and promote its degradation, although cytoplasmic arrestin-2 is sufficient to activate Mdm2 via the Akt pathway upon b2AR stimulation (Hara et al. 2011).

The ability of arrestins to engage the survival mechanisms via arrestin-dependent signaling could be taken advantage of via so-called biased ligands that are able to stimulate arrestin recruitment upon binding to GPCRs without inducing G protein activation (see chapter "Quantifying Biased β-Arrestin Signaling").

In some pathological conditions, the activity of select GPCRs is harmful, and in such cases antagonists are used as therapeutic agents. However, arrestin-dependent signaling, which might be beneficial, is also abolished by such drugs. The use of biased ligands achieves both ends: suppresses G protein-mediated and engages arrestin-mediated signaling. Thus, arrestin-biased ligand of angiotensin II type 1 receptor confers protection against cardiac injury induced by ischemia reperfusion injury or mechanical stretch, which is superior to that provided by angiotensin II antagonist losartan, a commonly used therapeutic agent. The protection was arrestin dependent, since it was absent in mice lacking arrestin-3 (Kim et al. 2012a).

4 Arrestins Regulate Apoptosis via Direct Interference in the Core Apoptotic Machinery

Thus, arrestins can affect cell survival in many ways via signaling, but direct pro-apoptotic action of arrestins at the core of cell death machinery was only recently reported (Kook et al. 2013). The signaling in the intrinsic apoptotic pathway involves cytochrome C release from the mitochondria that is orchestrated by the complex interplay of pro- and antiapoptotic members of the BCL-2 family of proteins (Chipuk and Green 2008, 2009). However, the exact mechanism of the process remains elusive. The involvement of additional players that do not belong to BCL family is one of the emerging ideas (Chipuk and Green 2008). Proteomic surveys suggest that caspase cleavage might supply regulators of apoptosis (Dix et al. 2008; Mahrus et al. 2008), but specific functional roles of cleavage products are rarely established. Active caspases are a notable example of caspase cleavage products playing critical role in cell death (Wolan et al. 2009). Another well-known example is BID: the product of its cleavage by caspases tBID translocates to mitochondria and promotes cytochrome C release. Possibly, some of the so-called dependence receptors require caspase-mediated cleavage for their death domains to be revealed or released (Bredesen et al. 2005). However, the full signaling potential of caspase cleavage products to affect this crucial step in the apoptotic pathway has not yet been explored.

As it turned out, arrestin-2 is cleaved by caspases at evolutionarily conserved Asp380 yielding an Arr2-(1-380) fragment (Kook et al. 2013). Apoptosis initiated via extrinsic (stimulation with TNFα combined with inhibition of protein synthesis by cycloheximide) or intrinsic (genotoxic drug etoposide) pathway resulted in the appearance of the same arrestin-2 fragment. A secondary cleavage site in arrestin-2, at Asp406 that is conserved only in mammals, was identified. When both aspartates were mutated to glutamates, the mutant (DblE) was resistant to caspases in all cell types (Kook et al. 2013). The presence of Asp380 in homologous positions in arrestin-2 from multiple species indicates that this mechanism is conserved in vertebrate evolution. Unlike many substrates, arrestin-2 is not just an “innocent victim” of caspases. 1-380 translocated to the mitochondria and enhanced

cytochrome C release by "assisting" another product of caspase-8 activity, tBID. Since virtually every mammalian cell expresses both arrestin-2 and arrestin-3 (Gurevich and Gurevich 2006), specific functions of individual subtypes can only be dissected in cells lacking one or the other. Arrestin-2 (A2KO) and arrestin-3 (A3KO) knockout mouse embryonic fibroblasts (MEFs), as well as arrestin-2/arrestin-3 double-knockout (DKO) MEFs, established more than a decade ago (Kohout et al. 2001), proved to be extremely useful tools in this regard. Increased cytochrome C release due to 1-380 significantly accelerated the progression of apoptosis. The rate of caspase activation and cell death in A3KO MEFs expressing only arrestin-2 was two- to threefold higher as compared to DKO MEFs lacking both arrestins. The ectopic expression of 1-380 in DKO MEFs facilitated TNF-α-induced apoptosis to the level observed in A3KO MEFs. WT arrestin-2 but not its uncleavable mutant also rescued vulnerability of DKO MEFs to cell death (Kook et al. 2013). Arrestin-2 does not have an identifiable mitochondrial localization signal, and mitochondria contain very little full-length arrestin-2, but large proportion of 1-380 localizes to mitochondria. Direct binding of purified 1-380 to isolated mitochondria and mitochondrial localization of expressed 1-380 even in non-apoptotic cells shows that, in contrast to full-length arrestin-2, it has an increased affinity for protein(s) residing in this compartment. 1-380 did not induce cytochrome C release by itself in cells or isolated mitochondria. Instead, it directly interacted with tBID and specifically facilitated cytochrome C release induced by tBID. The absence of BID completely abrogated pro-apoptotic effect of 1-380. Thus, caspase cleavage of arrestin-2 is a gain-of-function event resulting in a stronger interaction with tBID and the ability to enhance tBID-induced cytochrome C release that uncleaved arrestin-2 does not possess (Kook et al. 2013) (Fig. 1).

Both 1-380 and tBID are effectively generated by caspase-8, suggesting that their convergence at mitochondria plays crucial role in the extrinsic apoptotic pathway. However, apoptosis, like most cellular processes, has multiple backup mechanisms (Slee et al. 2000; Crawford and Wells 2011). Although the canonical way for caspase-8 activation is via death receptors, caspase-8 can also be activated in death receptor-independent manner, as seen, for example, in genotoxic drug-induced apoptosis (von Haefen et al. 2003; de Vries et al. 2007). Such activation occurs downstream of the mitochondria, cytochrome C release, and activation of effector caspases. Furthermore, in the absence of caspase-8, 1-380 could be generated by other caspases such as caspase-9 or caspase-6 (Kook et al. 2013).

Caspase activity in the cell is greatly increased by released cytochrome C via the apoptosome (Riedl and Salvesen 2007). Thus, cooperation of 1-380 and tBID in cytochrome C release creates a potent positive feedback loop, tipping the balance towards cell commitment to apoptotic death. This mechanism also sets a threshold for an irreversible cell "decision" to die: simultaneous generation of both fragments is necessary to maximize the death signal. The arrestin-2-dependent positive feedback loop greatly contributed to the mitochondrial apoptotic pathway, with magnitude of 1-380 effect on isolated mitochondria and intact cells comparable to that of tBID (Kook et al. 2013). The permeabilization of the outer mitochondrial membrane and the resulting cytochrome C release is usually the point of no return,

committing the cell to death (Danial and Korsmeyer 2004). Extensive studies of this step focusing on the interactions of pro- and antiapoptotic BCL family members with each other and pore-forming effectors BAK and BAX suggest that BID, BIM, and PUMA act as direct activators (Wei et al. 2000; Kim et al. 2009a; Ren et al. 2010). However, many molecular details necessary for mechanistic understanding of this process are missing (Chipuk and Green 2008; Youle and Strasser 2008). Our recent finding of the role of arrestin-2 cleavage product (Kook et al. 2013) supports the idea that direct involvement of additional players may explain inconsistencies between in vitro studies with BCL proteins and in vivo apoptosis (Chipuk and Green 2008). Our results suggest that tBID in complex with 1-380, rather than tBID alone, is the biologically relevant inducer of cytochrome C release (Kook et al. 2013). It is tempting to speculate that in cytochrome C release BIM and PUMA might also have their specific "helpers," possibly generated by caspases.

Caspases often produce discrete stable cleavage products likely serving as functional effectors in apoptosis (Dix et al. 2008; Mahrus et al. 2008). However, the functions of caspase-generated fragments are rarely established. The functional consequences of the cleavage of most of the 777 caspase substrates in CASBAH database (Lüthi and Martin 2007) remain unknown. Caspase cleavage of several kinases unleashes or abrogates their pro-apoptotic or pro-survival functions, respectively, via changes in activity, subcellular localization, or substrate preferences (Kurokawa and Kornbluth 2009). Caspase cleavage products of diverse proteins contribute to the progression of apoptosis due to loss or gain of function or via dominant-negative action (Kim et al. 2009b; Crawford and Wells 2011; Oliver et al. 2011). Our experiments revealed a direct role of 1-380 in cytochrome C release, identifying it as an earlier unappreciated active participant in the core mechanism of apoptosis (Kook et al. 2013). This is the first example of direct cooperation of two caspase products, 1-380 and tBID, at the point where the cell makes a fateful decision to live or die. This cooperation likely contributes to making this decision irreversible and also effectively sets a threshold for cell commitment to apoptotic death.

5 Visual Arrestins in Apoptosis

Of the four vertebrate arrestin isoforms, two, arrestin-1 (a.k.a. visual or rod arrestin) and arrestin-4 (a.k.a. cone arrestin), are expressed in retinal photoreceptors. Arrestin-1 is expressed in both rods and cones, whereas arrestin-4 is found in cones (Gurevich and Gurevich 2009, 2010; Gurevich et al. 2011). Photoreceptors are highly specialized neurons adapted for their function. Both rods and cones have specialized compartment, the outer segment, where photopigment and proteins of the signaling cascade reside, largely separated from the rest of the cell (Pugh and Lamb 2000). Rods function in dim light and are exquisitely light sensitive, being capable of detecting one photon of light (Baylor et al. 1979). Such sensitivity is

achieved, among other things, by high levels of expression of main signaling proteins such as photosensitive pigment rhodopsin (~3 mM) and arrestin-1 (>2 mM), an important component of the potent shutdown system ensuring almost zero background signaling (Pugh and Lamb 2000; Gurevich and Gurevich 2009; Gurevich et al. 2011). For comparison, the concentrations of higher expressed nonvisual arrestin isoform arrestin-2 in the adult rat central nervous system are ~200 nM and that of arrestin-3—almost 20-fold lower—~12 nM (Gurevich et al. 2004). Because of such high load of signaling proteins, the balance in rods is very precarious, and changes in the expression levels of signaling proteins often lead to rod death. A well-known example is rhodopsin: an excellent correlation between the level of overexpression of this perfectly normal protein and the rate of photoreceptor degeneration was established (Tan et al. 2001). Transgenic overexpression of wild-type arrestin-1 did not undermine photoreceptor survival, although it somewhat compromised the health of their outer segments in older mice (Song et al. 2011). Conversely, the loss of arrestin-1 induced defect in signaling shutoff, excessive signaling, and light-dependent degeneration of rod outer segments and rod death by apoptosis (Xu et al. 1997; Song et al. 2009b, 2013). Even hemizygous mice with ~50 % level of arretin-1 showed somewhat lower level of rod survival (Song et al. 2009b, 2013). Interestingly, the expression of arrestin-1 as low as 5 % of wild-type (WT) level was sufficient to maintain photoreceptor health and support their functional performance (Cleghorn et al. 2011; Song et al. 2011). Thus, the total loss of arrestin-1 function of quenching rhodopsin signaling is detrimental for rod survival and leads to rod death by apoptosis.

Arrestin interaction with phosphorylated rhodopsin that quenches phototransduction is required for rod survival. However, a very tight arrestin-rhodopsin interaction could be detrimental for rods, resulting in rod death. Such tight interaction is believed to cause retinal degeneration, a group of retinal degenerative diseases known as *retinitis pigmentosa* characterized by variable loss of rod photoreceptors across the retina followed by the death of cone photoreceptors (Mendes et al. 2005). Most of the cases are autosomal dominant and are caused by mutations in rhodopsin, leading to it being constitutively active or constitutively phosphorylated by rhodopsin kinase, with both conditions resulting in persistent arrestin-1 binding (Rim and Oprian 1995). Arrestin-1 mislocalizes rhodopsin from the outer segments to endosomes in inner segments and cell bodies, leading to rod death (Chuang et al. 2004; Chen et al. 2006). Recruitment of endocytic adapter protein-2 (AP-2) via arrestin-1 plays a role in rod death induced by arrestin-1 complex with constitutively active rhodopsin mutant (Moaven et al. 2013). A naturally occurring splice variant of arrestin-1 p44 lacking a part of the arrestin-1 C-tail and thus incapable of interacting with AP-2 but competent to quench phototransduction prevents the death of photoreceptors expressing constitutively active rhodopsin (Moaven et al. 2013). This pathway is evolutionarily conserved, since the same tight association of arrestin with activated rhodopsin induces apoptotic death of *Drosophila* photoreceptors (Alloway et al. 2000; Kiselev et al. 2000; Kristaponyte et al. 2012).

Arrestin-1 of several species has recently been shown to cooperatively form dimers and tetramers, with only a small fraction of it existing as monomer at physiological concentrations (Schubert et al. 1999; Imamoto et al. 2003; Hanson et al. 2007b, 2008b; Kim et al. 2011; Chen et al. 2013) (see chapter "Self-Association of Arrestin Family Members"). The physiological function of this phenomenon remained unclear. However, the mouse line expressing arrestin-1 mutant with reduced ability to form oligomers at ~240 % of WT arrestin-1 (which resulted in the monomer concentration exceeding that in WT mouse almost threefold) demonstrated rapid age-related apoptotic death of rod photoreceptors (Song et al. 2013). The mouse line expressing the same mutant at a much lower level (50 % of wild type, which yielded only ~20 % increase in monomer concentration) showed a very slow age-dependent degeneration. Importantly, mice overexpressing WT arrestin-1, which robustly oligomerizes, so that overexpression leads to minimal increase in the monomer concentration, did not show photoreceptor death. Furthermore, co-expression of WT arrestin-1 with the mutant protected rods from the mutant-induced apoptosis, suggesting that previously demonstrated ability of WT to recruit mutant arrestin-1 into mixed oligomers (Hanson et al. 2007b, 2008b) may be responsible. These data suggest that monomeric arrestin-1 is toxic to rods and provides a functional explanation for the ability of arrestin-1 to oligomerize. Only monomeric arrestin-1 interacts with rhodopsin (Hanson et al. 2007b, 2008b), and it binds rhodopsin monomer with high affinity (Hanson et al. 2007a; Bayburt et al. 2011; Kim et al. 2012b; Singhal et al. 2013; Vishnivetskiy et al. 2013; Zhuang et al. 2013). Thus, arrestin-1 oligomers likely represent a nontoxic storage form not only in rods but also in cones. Arrestin-4, or cone arrestin, in spite of its name, is outnumbered in cone photoreceptors by arrestin-1 by ~50:1 (Nikonov et al. 2008). Since cone arrestin is unable to self-associate (Hanson et al. 2008a), and as monomer could be toxic to photoreceptors, cones simply cannot afford to express arrestin-4 at high level necessary to rapidly quench phototransduction in bright light in which they operate. Therefore, a certain amount of arrestin-4 might be produced for immediate use, but the main stock is kept as arrestin-1 oligomers to be employed when needed.

In retinal photoreceptors, rhodopsin is the main binding partner of visual arrestins, and the main function of arrestins in photoreceptors is to quench rhodopsin signaling (Gurevich et al. 2011). However, arrestin-1 is found not only in close proximity to its key binding partner rhodopsin, i.e., in the rod outer segments, but also in other rod compartments, specifically in synaptic terminals (Nair et al. 2005; Hanson et al. 2007a; Huang et al. 2010). Very little attention so far has been paid to possible rhodopsin-independent functions of arrestin-1 and their potential role in retinal photoreceptor death and survival. It has recently been shown that arrestin-1 interacts with N-ethylmaleimide-sensitive factor (NSF) (Huang et al. 2010). NSF is localized to photoreceptor synapses and functions to sustain high rate of neurotransmitter exocytosis. Arretin-1 interaction with NSF was enhanced in the dark when rods were depolarized and neurotransmitter release was elevated. Mice lacking arrestin-1 displayed reduced levels of NSF and of other synaptic proteins, as well as reduced exocytosis, suggesting that arrestin-1 interaction with NSF was required for normal synaptic function in rods. Therefore, it is conceivable that

arrestin-1 modulates rod survival via its interactions with proteins other than rhodopsin, such as synaptic proteins. It has been shown that arrestin-1 mutant with reduced ability to self-associate caused damage to synaptic terminals, which was detectable earlier than the loss of photoreceptors, suggesting that synapses might be the site of toxicity of arrestin-1 monomer (Song et al. 2013). Co-expression of WT arrestin-1 that protected photoreceptors from apoptosis conferred even more significant protection to synapses, again supporting the notion of synapses being the primary site of damage. These data indicate that the role of arrestin-1 in the photoreceptor death and survival could involve arrestin-1 interaction with proteins other than rhodopsin. Specifically, arrestin-1 function in photoreceptor synapses could be necessary to maintain photoreceptor health. Interestingly, the binding of arrestin-2 to NSF was described more than a decade ago (McDonald et al. 1999), suggesting that nonvisual subtypes might also be involved in synaptic functions, including neurotransmitter release.

6 Conclusions and Future Directions

Apoptotic cell death plays an important role in embryonic development, in homeostasis of multicell organisms, as well as in numerous pathological processes. Arrestins appear to be intimately involved in the regulation of a variety of signaling pathways involved in cell death and survival. Thus, reengineered signaling-biased arrestins with enhanced pro-apoptotic or pro-survival functions can be used as molecular tools to influence cell decision to live or die in desired direction (Gurevich and Gurevich 2012). Arrestin-3 mutants lacking the ability to activate JNK family kinases (chapter "Arrestin-Dependent Activation of JNK Family Kinases") are obvious candidates to be tested in this regard. In addition, caspase cleavage product of arrestin-2 directly participates in the critical step in vertebrate apoptosis, assisting tBID in cytochrome C release (Kook et al. 2013). Importantly, caspase-resistant arrestin-2 mutant is lacking pro-apoptotic function (Kook et al. 2013), suggesting that it might be useful for cytoprotection. This finding also raises a question whether other arrestin subtypes, such as arrestin-1 and arrestin-3, are targeted by caspases and whether generated cleavage products acquire new functions, similar to that of arrestin-2. Visual arrestin-1, the functions of which were long believed to be limited to the shutoff of rhodopsin signaling (Gurevich et al. 2011), turned out to be an important regulator of photoreceptor health and survival acting at synaptic terminals (Song et al. 2013). Since the integrity of synapses is necessary for survival of all neurons, nonvisual arrestins might play a role in neuronal survival similar to that described for arrestin-1. Three out four vertebrate arrestin subtypes self-associate forming distinct oligomers (Chen et al. 2013). Since arrestin-1 monomers appear to be cytotoxic, whereas oligomers are perfectly harmless (Song et al. 2013), self-association of nonvisual arrestins might also affect their role in cell survival (see chapter "Self-Association of Arrestin Family Members"). Considering that arrestins can impact cell death and

survival via numerous mechanisms, every redesigned arrestin mutant, including those with enhanced ability to bind unphosphorylated receptors (chapter "Enhanced Phosphorylation-Independent Arrestins and Gene Therapy"), targeting individual GPCRs (chapter "Targeting Individual GPCRs with Redesigned Non-visual Arrestins"), and with modified trafficking properties (chapter "β-Arrestins and G Protein-Coupled Receptor Trafficking"), changing interactions with ubiquitinating and deubiquitinating enzymes (chapter "Arrestin Interaction with E3 Ubiquitin Ligases and deubiquitinases: Functional and Therapeutic Implications"), must be specifically tested for its effect on apoptosis and cell survival.

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Molecular Mechanisms Underlying Beta-Arrestin-Dependent Chemotaxis and Actin-Cytoskeletal Reorganization

Kathryn W. McGovern and Kathryn A. DeFea

Contents

Abstract β-Arrestins play a crucial role in cell migration downstream of multiple G-protein-coupled receptors (GPCRs) through multiple mechanisms. There is considerable evidence that β-arrestin-dependent scaffolding of actin assembly proteins facilitates the formation of a leading edge in response to a chemotactic signal. Conversely, there is substantial support for the hypothesis that β-arrestins facilitate receptor turnover through their ability to desensitize and internalize GPCRs. This chapter discusses both theories for β-arrestin-dependent chemotaxis in the context

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of recent studies, specifically addressing known actin assembly proteins regulated by β-arrestins, chemokine receptors, and signaling by chemotactic receptors.

Keywords Actin • Arrestins • Chemokine receptor • Chemotaxis • GPCR • Cell migration

1 β-Arrestins as Regulators of Cell Migration: Receptor Turnover Versus Scaffolding of Actin Assembly Proteins

β-Arrestins are required for cell migration downstream of numerous receptors and have been implicated in a number of physiological scenarios involving cell migration, including tumor cell metastasis, inflammation, neuronal synapse remodeling, and developmental patterning (Min and Defea 2011). Given their essential roles in G-protein-coupled receptor (GPCR) signaling and signal termination, there are currently several hypotheses regarding their role in cell migration. Based on their ability to uncouple G-proteins from GPCRs and promote receptor internalization, some investigators propose that β-arrestins are crucial in receptor turnover at the leading edge, a process that is important for a cell's ability to sense a gradient of agonist and migrate toward it. However, the ability of β-arrestins to scaffold signaling proteins and actin assembly machinery at the leading edge suggests that their signaling functions are also key factors in many cell migratory pathways. Thus, it is likely that the ability of β-arrestins to control both receptor turnover and actin assembly at the leading edge is crucial for cell migration (Fig. 1). Furthermore, the emergence of β-arrestin-biased agonists for a number of GPCRs suggests that it is possible to target β-arrestin signaling without G-protein engagement (see chapter "Quantifying Biased β-Arrestin Signaling"). Given the importance of β-arrestins in numerous diseases involving cell migration, the therapeutic implications of β-arrestin-biased drugs are very promising.

1.1 Regulation of Receptor Turnover

We will first look at the model wherein β-arrestins regulate turnover of receptor and chemokine to maintain a gradient of agonist and an active pool of receptors. The first argument in favor of this model is that β-arrestins play a well-known role in clathrin-mediated endocytosis of numerous GPCRs, acting as adaptors for clathrin (Goodman et al. 1996) and clathrin adaptor AP2 (Laporte et al. 1999) (see chapter "β-Arrestins and G Protein-Coupled Receptor Trafficking"). They also uncouple GPCRs from their cognate heterotrimeric G-proteins, thus rendering them

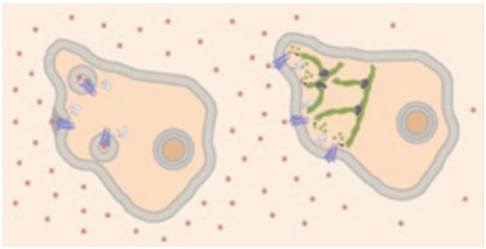

Fig. 1 Models for β-arrestin-dependent chemotaxis. As a cell senses a chemotactic gradient (depicted as *red circles*), it polarizes in the direction of the gradient. Current models predict that at low concentrations of agonist, β-arrestin-dependent receptor recycling and ligand degradation, paired with scaffolding and activation of actin assembly proteins, allows the cell to sense the chemotactic gradient and form a leading edge to direct chemotaxis. At high concentrations of agonist, receptor and ligand are degraded, ceasing the migratory process

insensitive to further agonist stimulation (DeWire et al. 2007; Shenoy and Lefkowitz 2011). During chemotaxis (directed cell migration) in vivo, a cell senses gradient of a chemotactic agonist; it essentially migrates up the gradient and stops when it reaches the source, i.e., where the concentration is highest. A second important factor in successful cell migration in vivo is the ability of the cell to stop migrating when it reaches a uniform concentration of agonist, a phenomenon that prevents cells from migrating back away from their destination (Iglesias and Devreotes 2008). Because prolonged exposure to any agonist usually results in downregulation of receptors, it has been proposed that gradient-sensing chemotactic receptors are able to rapidly turnover at the leading edge such that the cell remains responsive to the chemoattractant (Fig. 2). This receptor recycling appears to require β-arrestins in many cases. Upon internalization, β-arrestins either dissociate in the early endosomes allowing recycling of the receptor and degradation of the agonist or remain associated and target the receptor to lysosomes for degradation. In some cases, β-arrestins can also remain bound to the receptor and recruit signaling proteins to form a "signalosome" (discussed in the next section; see also chapters "Arrestin-Dependent Activation of ERK and Src Family Kinases," "Arrestin-Dependent Activation of JNK Family Kinases," and "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences") or facilitate their degradation, through interaction with ubiquitin ligases (chapter "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications") or targeting to lysosomes. By allowing receptors to recycle back to the membrane and facilitating removal of agonists, it is predicted that transient interactions with β-arrestins contribute to the maintenance of both a chemotactic gradient and a pool of responsive receptors. At very high concentrations of agonist (such as would be seen at the cells "destination") (Fig. 2), it is proposed that β-arrestins target endocytosed receptors for degradation through ubiquitin- and lysosomal-mediated processes. This model would predict that β-arrestin/receptor interactions vary in an agonist dose-dependent fashion.

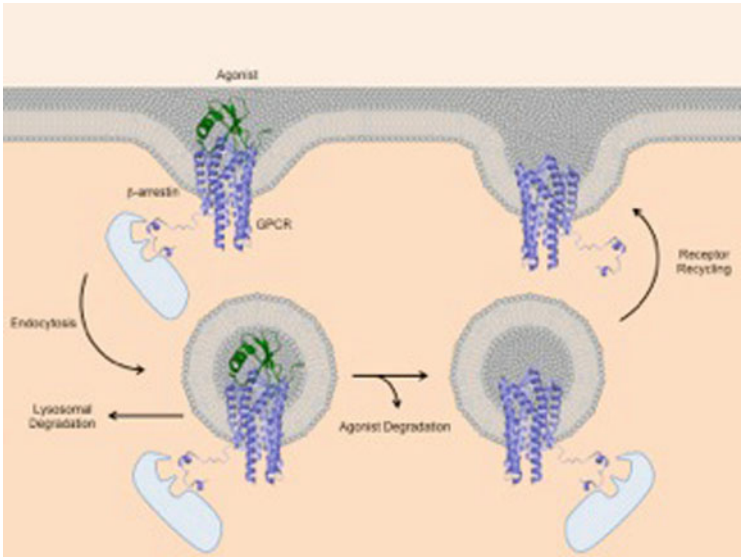

Fig. 2 Trafficking of receptor and chemokine by β-arrestins. In the receptor turnover/ligand degradation model, β-arrestins bind chemotactic receptors, to promote clathrin-dependent internalization of the receptor. In the early endosome, receptors dissociate from ligand and β-arrestin, allowing for the receptor to be returned to the surface and the ligand degraded. This model predicts that because β-arrestins are required to target chemokine-bound receptors to clathrin-coated pits, in their absence, receptors remain at the surface and chemokine levels accumulate. This results in the cell losing polarity and being unable to migrate

There are considerable data to support this receptor turnover/agonist degradation model for β-arrestin-dependent chemotaxis. Early studies investigating the role of β-arrestins in chemotaxis revealed that many of the receptors that require β-arrestins for internalization also show impaired chemotaxis in their absence (DeFea 2007). Receptors that were allowed to signal constitutively, such as they would in the absence of β-arrestins, also showed impaired chemotaxis. Recent proteomics screens for β-arrestin-binding proteins have led to the identification of more clathrin adaptor proteins, such as AGEP and ARF, with additional evidence suggesting that chemokine receptor turnover via β-arrestins, through association with endocytotic machinery, is required for migrating cells to respond to an agonist gradient (Bouschet et al. 2007; DeFea 2007; Xiao et al. 2007). Biochemical isolation of pseudopodia (actin-rich extensions formed at the front of a migrating cell) reveals that β-arrestins and other components of the endocytotic machinery are enriched at the leading edge (Ge et al. 2003; Parisis et al. 2013). Furthermore, early data suggested that coupling to $G\alpha_{12}$ or $G\alpha_q$ was required for chemotaxis downstream of many of these chemotactic receptors. Collectively, these data have led to the hypothesis that G-protein signaling mediates the chemotactic response, while β-arrestin-dependent turnover of receptors and chemokine at the leading edge is important for maintaining responsiveness to the chemoattractant gradient.

However, a number of receptors can promote β-arrestin-dependent cell migration in the absence of prior G-protein coupling, suggesting a more complex paradigm is necessary to explain this process. Since most commonly used assays for quantifying chemotaxis do not differentiate effectively between differences in migration speed, persistence, and directionality, it is likely that these more subtle aspects of cell migration require the cooperation between β-arrestin signaling, G-protein signaling, and β-arrestin-dependent receptor turnover.

1.2 Regulation of Actin Assembly

We will now examine the model in which β-arrestins facilitate chemotaxis by sequestering and activating actin assembly proteins at the leading edge. A number of studies have demonstrated a role for β-arrestins in the modulation of actin assembly, the process that provides the primary driving force behind the initial formation of a leading edge. Actin assembly can be regulated, both directly and indirectly, by various proteins, some of which have been identified as β-arrestin-binding partners or as targets of β-arrestin-dependent phosphorylation (Xiao et al. 2007, 2010; Christensen et al. 2010; Min and Defea 2011). To understand the role of β-arrestins in the initiation of cell migration, it is important to understand the mechanics of actin assembly. Actin polymerization from monomers is a spontaneous but slow process, and the rate-limiting step is the formation of a stable nucleus, or actin seed, consisting of three or more actin monomers. Addition of actin monomers is always at the ATP-binding or barbed end of the actin molecule (Campellone and Welch 2010). Provided that the barbed end of a filament is free from capping proteins, addition of monomers onto preassembled filaments or actin seeds is very rapid. These seeds can be created in two major ways: (1) activation of proteins that sever existing filaments into smaller fragments creating a free barbed end at each break or (2) activation of nucleators, i.e., proteins that overcome the rate-limiting step in actin assembly by facilitating association of actin monomers into filament seeds. Actin nucleators can also bind to the sides of existing filaments, an action that in turn facilitates branching of actin filaments. These branched filaments are found in the broad lamellipodia of the leading edge (Firat-Karalar and Welch 2011). Together, these processes provide directionality during migration, as well as assemble and maintain cortical actin filaments. Cortical actin filaments interact with contractile proteins and allow the cell to contract against the substrate over which it is migrating (Campellone and Welch 2010). These actin structures must be dynamically remodeled, requiring an intricate balance of input from various signaling pathways.

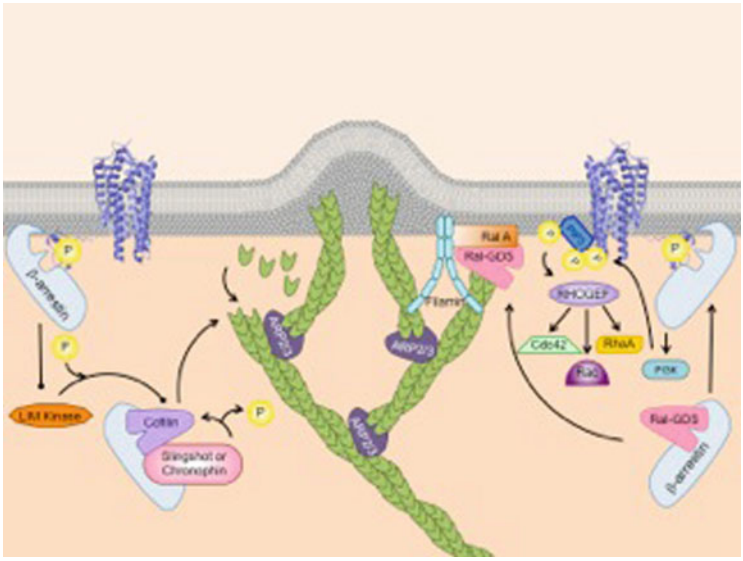

Fig. 3 Scaffolding of signaling molecules by β-arrestins at the leading edge. Many signaling molecules are sequestered at the leading edge by β-arrestins in response to a chemotactic signal. β-Arrestins have been shown to bind and inactivate LIMK while scaffolding cofilin with its upstream phosphatases. Together these events result in increased actin filament severing, creating of free actin barbed ends and actin polymerization. Proteins associated with ARP2/3-mediated nucleation have been identified as targets of β-arrestin-dependent phosphorylation. Active cofilin and Arp2/3 work together to create branched lamellipodia that push the membrane forward during chemotaxis. β-Arrestins have also been demonstrated to bind filamin and regulate activation of the G-protein Ral, which is important for membrane protrusion formation. Finally, β-arrestins regulate the cellular pool up PIP3 through activation and inactivation of PI3K. This controls the activation of many guanine exchange factors for RhoA, Cdc42, and Rac, providing a spatiotemporal regulation of these small G-proteins and, in turn, their ability to promote stress fiber, lamellipodia, and filopodia formation

1.2.1 Regulation of the Cofilin Pathway

A requirement for β-arrestin in the creation of free barbed ends through cofilin-mediated actin filament severing strongly supports the notion that β-arrestins regulate actin assembly (Zoudilova et al. 2007, 2010). Cofilin is one of the primary actin filament-severing proteins and its activation is often an early event in cell migration. Cofilin is activated by dephosphorylation on serine 3, carried out by the phosphatases slingshot and chronophin, and inactivated by LIM kinase (LIMK). Its activity is crucial to the formation of a leading edge, downstream of multiple receptors (Wang et al. 2007; Oser and Condeelis 2009). Several lines of evidence indicate that proteins of the cofilin pathway (cofilin, chronophin, slingshot, and LIMK) can interact with β-arrestins. β-Arrestins can facilitate cofilin dephosphorylation by scaffolding it with its activating phosphatases at the leading edge or by binding and inhibiting LIMK-induced phosphorylation (Zoudilova et al. 2007,

2010; Xiao et al. 2010). A popular model for actin remodeling at the leading edge predicts that cofilin rapidly disassembles existing filaments, providing free barbed ends for elongation and, in coordination with activation of nucleating proteins, drives the direction of cell migration (Oser and Condeelis 2009). Spatial control over cofilin activity is thus essential to allow for treadmilling of filaments. This model, combined with data demonstrating β-arrestin-dependent cofilin activation and sequestration (Zoudilova et al. 2007, 2010), suggests that β-arrestins spatially regulate cofilin activity. By restricting cofilin activity to the front of the migrating cell, they can facilitate protrusion formation while preventing severing of the more stable cortical actin filaments. Several studies showing β-arrestin-dependent localization of cofilin and cofilin activity support this hypothesis (Zoudilova et al. 2010; Pontrello et al. 2012). A scaffold containing β-arrestin, cofilin, and chronophin has been demonstrated in primary leukocytes and is essential for their migration (Zoudilova et al. 2010). When cofilin activity is not spatially controlled, protrusions can form randomly and cells lose their ability to move toward a chemotactic gradient (Mouneimne et al. 2004). Thus, it is likely that β-arrestins contribute to the formation of a leading edge, through the regulation of cofilin activity (Fig. 3).

1.2.2 Filamin and Other Actin-Bundling Proteins

The actin-binding protein, filamin, has been identified in a complex with β-arrestins and ERK1/2. The role of filamin in cell migration is a compound one, and the manner in which it is controlled by β-arrestins is still not completely clear. It plays an important role in turnover of adhesion proteins such as integrins during cell migration (Kim and McCulloch 2011), a process that is important for cell attachment and contraction. It has also been reported to regulate the internalization of GPCRs such as the dopamine receptor (Kim et al. 2005; Cho et al. 2007) and CXCR4 (Kim and McCulloch 2011). Downstream of angiotensin II receptor (AT1AR), filamin associates with β-arrestin-2 and MAPK and this complex is thought to play a role in membrane ruffling (Scott et al. 2006). These studies support both models of β-arrestin involvement in chemotaxis. First, the studies on dopamine receptor and CXCR4 support a model where β-arrestin-dependent regulation of filamin contributes to both focal adhesion remodeling and receptor turnover, both of which are proposed to be important for gradient sensing. However, the studies on AT1AR suggest that β-arrestin-bound filament is involved in the sequestration of ERK1/2 activity at the leading edge (Ge et al. 2003), which likely facilitates localized phosphorylation of key substrates involved in chemotaxis.

While a number of actin nucleators and accessory proteins have been identified as either putative β-arrestin-binding partners or substrates for β-arrestin-dependent phosphorylation, regulation of actin nucleation has not yet been demonstrated for β-arrestins. However, it remains possible that some of these proteins may be among the long sought-after substrates for β-arrestin-sequestered MAPK activity. This possibility is discussed in the sections below.

2 Chemokine Receptors: The Classic Chemotactic Receptors

The most well-characterized mediators of chemotaxis are the chemokine receptors, a family of G-protein-coupled receptors that play crucial roles in immune cell recruitment and tumor cell metastasis. β-Arrestins are required for the desensitization and internalization of many chemokine receptors, including CXCR4, CXCR7, CXCR1 and 2, CXCR5, CCR2, CCR5, and CCR7. For CCR2, CCR5, and CXCR5, β-arrestin recruitment has been reported, but little is known about the role of β-arrestins in their chemotactic responses. Because they are well-established mediators of cell migration, we will examine some examples of signaling through β-arrestins downstream of some of these chemokine receptors. In the case of CXCR4 and 7, CXCR1 and 2, and CCR7, recent studies have revealed multiple roles for β-arrestins in their signaling pathways.

CXCR4 is a crucial mediator of cell migration in many cells: monocytes/macrophages, T cells, B cells, plasma cells, neutrophils, and dendritic cells, as well as tumor cells and germ cells. It was one of the first to show impaired cell migration in the absence of β-arrestin-2 (Fong et al. 2002). CXCR4 cooperates with another chemokine receptor CXCR7 in a unique fashion. CXCR7 is considered a "scavenger" receptor for CXCR4 ligands, as it binds CXCL12 without promoting G-protein signaling. Both CXCR4 and CXCR7 recruit β-arrestins, and internalization of both is impaired in their absence (Malik and Marchese 2010; Rajagopal et al. 2010; Décaillot et al. 2011). Studies have predicted that the main role of CXCR7 in chemotaxis is the internalization of CXCL12, something that is dependent upon the presence of β-arrestins. Recent in vivo studies examining primordial germ cell migration in zebrafish suggested that, in the absence of β-arrestins, CXCR7 did not recycle to the membrane, but was targeted to lysosomes. As a result the gradient of the agonist CXCL12 was not maintained and germ cell migration was also inhibited (Décaillot et al. 2011). Thus, CXCR7 is a prime example of a receptor system that utilizes β-arrestins for gradient sensing by reducing the immediate concentration of CXCL12, thus perpetuating the gradient. However, another recent study reveals that CXCR4 can also form heterodimers with CXCR7 and that this heterodimer preferentially promotes β-arrestin-dependent ERK1/2 phosphorylation and chemotaxis over G-protein signaling (Drury et al. 2011). This study would suggest that β-arrestin-dependent signaling is associated with chemotaxis. Thus, the CXCR4/CXCR7 system stands out as an argument for the importance of both ERK1/2 scaffolding and internalization functions of β-arrestins in chemotaxis.

CXCR1 and CXCR2 are activated by CXCL8 (aka IL-8) in humans and CCL-1 (GRO/KC) in mice to promote recruitment primarily of neutrophils but also basophils and CD8+ T cells. It is also expressed on endothelial cells where it may play a role in extravasation. Both receptors promote chemotaxis but CXCR2 is internalized more rapidly in response to IL-8 (Barlic et al. 1999; Richardson et al. 2003). Internalization and degranulation are all induced by IL-8 via a

β-arrestin-dependent mechanism; however, the role of β-arrestins CXCR1- or CXCR2-stimulated migration depends upon the organism and the system studied. In mice, CXCL1-mediated neutrophil migration is enhanced, in vivo, in β-arrestin-$2^{-/-}$ mice (Su et al. 2005). In vitro, human neutrophil recruitment in response to IL-8 is abolished in the absence of β-arrestins (Barlic et al. 2000). Based on these opposite findings with respect to the role of β-arrestins in CXCR1- and CXCR2-induced chemotaxis, one might conclude that CXCR1- and CXCR2-induced migration in response to CXCL1 differs from that observed with the human CXCR1 and 2 agonist, IL-8. Alternatively, since the CXCL1 experiments were based on recruitment of neutrophils in vivo, while the IL-8 experiments were performed on isolated neutrophils, it is possible that additional factors contributing to chemotaxis may be differentially affected by β-arrestins in vivo.

CCR5 is widely expressed in immune cells, especially memory T cells and has an array of physiologically relevant agonists (RANTES, MIP-1α, MIP-1β, and MCP-2). In response to RANTES, CCR5 internalizes via a β-arrestin-dependent mechanism (Kraft et al. 2001). Another physiological agonist of CCR5, MIP1β, induces the formation of a membrane-associated multiprotein complex including β-arrestin-2, PI3K, and a number of non-receptor tyrosine kinases. Scaffolding of these kinases by β-arrestin-2 appears to be crucial for MIP1β-induced chemotaxis (Cheung et al. 2009). It is tempting to assume that RANTES and other CCR5 agonists would induce a similar β-arrestin scaffold-dependent mechanism of chemotaxis; however, given the emergence of naturally occurring biased agonists, it remains possible that different CCR5 agonists might use β-arrestin in distinct manners during chemotaxis.

CCR7 is a well-established homing receptor, expressed on mature dendritic cells (DCs), naïve and memory T cells, and naïve B cells. CCR7 has two ligands, CCL-19 and CCL-21, which bind to their receptor with equal affinity, and are equally efficacious at promoting Ca^{2+} mobilization (Yoshida et al. 1998; Sullivan et al. 1999). Despite their apparent similarities with respect to G-protein signaling, subtle differences elicited by CCL-19 and CCL-21 have been reported (Yoshida et al. 1998; Sullivan et al. 1999; Ott et al. 2004). In contrast to Ca^{2+} mobilization, ERK1/2 activation in response to CCL-19 is more robust than in response to CCL-21. Knockdown of β-arrestin-2 reduces ERK1/2 phosphorylation in response to CCL-19; whether it does the same in response to CCL-21 is not known. Similar to what was observed for ERK1/2 phosphorylation, both CCL-19 and CCL-21 can promote β-arrestin-2 recruitment to CCR7 but CCL-21 does so to a lesser degree than CCL-19 (Zidar et al. 2009). Further differences are observed with respect to receptor endocytosis. Depending on the cell type studied, very little or no endocytosis in response to CCL-21 is observed. In contrast, CCL-19 promotes receptor endocytosis in a variety of cell types (Bardi et al. 2001; Byers et al. 2008). Further, phosphorylation of CCR7 by GRK6 occurs in response to either ligand, but phosphorylation by GRK3 only occurs after stimulation with CCL-19 (Zidar et al. 2009). Thus, differences in GRK-mediated phosphorylation of CCR7 at the carboxy-terminal serine/threonine cluster in response to each agonist may underlie these differences in β-arrestin recruitment and subsequent receptor internalization

and MAPK activation (Kohout et al. 2004). However, whether differential phosphorylation of CCR7 recruits separates functional pools of arrestins has not been definitely proven.

3 β-Arrestin-Dependent Signaling by Chemotactic Receptors

A number of other GPCRs that promote β-arrestin-dependent chemotaxis, and while they do not fall into the category of chemokine receptors, they have provided important insights into how β-arrestins regulate various aspects of cell migration. In fact, the majority of what we know regarding β-arrestin-dependent signaling and reorganization of the cytoskeleton (a crucial factor in cell migration) has been mapped out using less typical chemotactic receptors. We have discussed, earlier in this chapter, how β-arrestins scaffold and regulate some actin assembly proteins to influence actin-cytoskeletal remodeling, but they also regulate a number of other signaling networks that impact the actin cytoskeleton indirectly. These include MAPKs, RhoA GTPases, and PI3K. MAPK was the first "β-arrestin-dependent signal" to be identified and there is considerable evidence that, when active MAPKs are sequestered by β-arrestins, they can phosphorylate substrates to facilitate actin reorganization (Ge et al. 2003, 2004).

3.1 β-Arrestin-Dependent ERK1/2 Activity

There is a significant body of evidence pointing to an essential role of for β-arrestin sequestration of ERK1/2 in chemotaxis; however, information as to what it these kinases are phosphorylating at the leading edge has been slow to emerge. Many of the receptors first demonstrated to promote β-arrestin-dependent ERK1/2 activation also require β-arrestins and/or ERK1/2 for chemotaxis. PAR_2-stimulated ERK1/2 activation requires β-arrestin-dependent endocytosis, and chemotaxis is dependent on both β-arrestins and ERK1/2 (DeFea et al. 2000; Ge et al. 2003). AT1AR also requires β-arrestins for both ERK1/2 activation and chemotaxis (Tohgo et al. 2002; Hunton et al. 2005). CXCR4 can form heterodimers with the decoy receptor CXCR7, leading to dominance of β-arrestin-dependent ERK1/2 phosphorylation and chemotaxis over G-protein signaling (Décaillot et al. 2011). Thus, CXCR4 and PAR_2 stand out as an argument for the importance of both ERK1/2 scaffolding and internalization functions of β-arrestins in chemotaxis. A number of proteins involved in actin assembly have been identified as β-arrestin-dependent ERK1/2 phosphorylation targets; however, for most of these a definitive role in chemotaxis downstream of β-arrestins has not been proven.

Regulation of actin nucleation by β-arrestins has not been directly shown, although several proteins involved in actin nucleation have been identified as β-arrestin-dependent ERK1/2 targets. Arp2/3 complex components and Wiskott–Adrich syndrome protein (WASp) family proteins have been identified in a proteomics screen as potential β-arrestin-interacting proteins (Xiao et al. 2007) and phosphorylation targets of MAPK (Christensen et al. 2010) and activation of WASp family proteins is enhanced by ERK1/2 phosphorylation (Mendoza Michelle et al. 2011). The Arp2/3 complex, along with formins and p150spir, is the primary nucleation factor in mammalian cells (Campellone and Welch 2010; Firat-Karalar and Welch 2011). Arp2/3 is crucial for the formation of branched actin filaments such as are observed in the leading edge of migrating cells. Activation by WASps, which bind Arp2/3 and induce a conformational change resulting in apposition of Arp2 and 3, is essential to formation of branched filaments. Patients with Wiskott–Aldrich syndrome have defective lymphocyte trafficking and function, due in part to the disruption of actin nucleation. The actin-nucleating proteins, formins, and p150spir are primarily responsible for de novo assembly of unbranched actin filaments (Firat-Karalar and Welch 2011). Recently, a formin-like protein was also identified as a putative β-arrestin-dependent phosphorylation target (Christensen et al. 2010). Thus, β-arrestins may sequester kinases such as ERK1/2 at the leading edge where they could promote actin nucleation through the phosphorylation of Arp2/3 and formins or by the phosphorylation and activation of WASp family proteins.

So far we have discussed actin assembly proteins that create free actin barbed ends by regulating actin nucleation or increasing filament severing. However, the pool of free barbed ends, available for polymerization, can also be increased by removing capping proteins. Another protein identified as a putative β-arrestin-dependent ERK1/2 substrate in two separate proteomics screens is the barbed-end capping protein adducin. Phosphorylation of adducin by Ser/Thr kinases, PKC, and Rho-activated kinase (ROCK) diminishes its affinity for actin, increasing the pool of free barbed ends and facilitating actin polymerization. Thus, phosphorylation by ERK1/2 may have a similar effect. Cofilin (discussed above) was also identified as a β-arrestin-dependent ERK1/2 substrate. The known regulatory site on cofilin (S3) was not the site identified in the phosphoproteomics screen. Thus, if ERK1/2 phosphorylation contributes to β-arrestin-dependent cofilin dephosphorylation, it would be through a distinct mechanism, possibly by stabilizing the active form.

3.2 β-Arrestin-Dependent Regulation of Src and Chemotaxis

Src was identified as a β-arrestin-binding partner downstream of the several GPCRs, linking them to ERK1/2 activation (Luttrell and Gesty-Palmer 2010) (see chapter "Arrestin-Dependent Activation of ERK and Src Family Kinases"). Downstream of prostaglandin E2 (PGE2) receptor, cell migration involves β-arrestin-dependent recruitment of Src into a signaling complex that then transactivates the

EGF receptor (Kim et al. 2010). Src and other non-receptor tyrosine kinases also impact cell migration through multiple mechanisms, including stabilization of nucleation-promoting factors in their active forms. In an example discussed earlier, we saw that activation of CCR5 with MIP1α led to recruitment of Pyk2, the p85 regulatory subunit of PI3K, and the tyrosine kinase, Lyn, into a complex with β-arrestin-2 that appears to be essential for macrophage chemotaxis (Cheung et al. 2009). There are a number of putative functions for membrane-recruited PYK2 and Lyn tyrosine kinase activities. Both have been implicated in activation of nucleation-promoting factors (Guinamard et al. 1998; Chellaiah et al. 2007). PI3K generates PIP3, which is necessary for activation of Cdc42 and Rac activators (Hall 1998). Just as β-arrestins sequester and activate MAPKs at the membrane, sequestration of Src-like kinases by β-arrestins may allow for phosphorylation of actin nucleation proteins and other components of cytoskeletal machinery to facilitate cell migration.

3.3 RhoA GTPases

RhoA GTPases are often upstream regulators of both cofilin and other actin assembly activities. The three most commonly studied members of the Rho family are RhoA, Rac-1, and Cdc42, and each is associated with a different actin structure: RhoA typically causes stress-fiber formation; Cdc42 induces filopodia formation; and Rac-1 is important for membrane ruffling and lamellipodia formation (Hall 1998). β-Arrestins can regulate the activity of many monomeric GTPases including those of the Rho family (Bhattacharya et al. 2002, 2006; Barnes et al. 2005) (see chapter "Arrestin Regulation of Small GTPases"). For example, knockdown of β-arrestin-1 but not β-arrestin-2 with siRNA significantly reduces RhoA activation and stress-fiber formation by the angiotensin receptor, AT1AR (Barnes et al. 2005). β-Arrestin-1-dependent p38 MAPK activation can elicit F-actin rearrangement via a Rac-1-dependent mechanism, downstream of β_2-adrenergic receptors (Gong et al. 2008), raising the possibility that Rac-1 could be a β-arrestin-dependent p38 MAPK target. β-Arrestins can also negatively regulate RhoA GTPases. The type III TGF-β receptor (TβRIII) forms a β-arrestin-2 scaffolding complex with Cdc42, leading to inhibition of lamellipodia formation in both cancer and epithelial cells (Mythreye and Blobe 2009a, b).

How is Rho GTPase activity affected by β-arrestins? To answer this question, one must consider how monomeric GTPases are regulated in general. Their activity is increased by binding to guanine exchange factors (GEFs) and turned off by association with GTPase-activating proteins (GAPs). β-Arrestin-1 directly binds and inhibits the RhoGAP (ARHGAP21) downstream of AT1AR activation. Disruption of this complex inhibits AT1AR-mediated stress-fiber formation and RhoA activation (Anthony et al. 2011). Phosphatidylinositol-3-phosphate (PIP3), which is generated by PI3K, can also activate a number of GEFs for RhoA, Cdc42, and Rac. β-Arrestins have been shown to regulate PI3K activity both positively and

negatively depending on the activating receptor (Povsic et al. 2003; Wang and DeFea 2006) (Fig. 3). β-Arrestin-1-dependent p38 MAPK activation was reported to elicit F-actin rearrangement via a Rac-1-dependent mechanism, downstream of β2-adrenergic receptors (Gong et al. 2008), and so regulation of RhoA GTPases may also lie downstream of the MAPK scaffolds. β-Arrestin-dependent regulation of RhoA GTPases has also been implicated in inhibition of cell migration. The type III TGF-β receptor (TβRIII) inhibits migration and alters actin cytoskeleton via forming a β-arrestin-2 scaffolding complex with Cdc42 in both cancer and epithelial cells (Mythreye and Blobe 2009a, b).

Another GTPase, RalA, induces membrane blebbing in response to the fMLP receptor in neutrophils and LPA in cancer cells (Bhattacharya et al. 2002, 2006; Li et al. 2009). RalGDS is a guanine exchange factor that activates RalA and can exist in an inactive complex with β-arrestin-1 in the cytosol of resting cells. Activation of the fMLP or LPA receptor recruits the β-arrestin-1/RalGDS complex to the membrane. Upon receptor/β-arrestin-1 binding, RalGDS is released and activates RalA. fMLP receptor-induced membrane ruffling is blocked by a mutant RalGDS which cannot bind β-arrestin-1, suggesting that the ability of β-arrestin-1 to traffic it to the membrane is crucial for its activity (Fig. 3). Subsequent studies have demonstrated that expression of RalA and β-arrestin-1 and β-arrestin-2 is increased in metastatic cancers and expression of a RalGDS mutant that is deficient in β-arrestin binding inhibits RalA activation in tumor cells, leading to decreased cell migration. RalA itself is important for targeting another actin-binding protein regulated by β-arrestins, filamin, to the membrane (Ohta et al. 1999).

4 Role of β-Arrestin-Dependent Chemotaxis in Health and Disease

β-arrestin-dependent chemotaxis has been implicated in both tumor cell metastasis and inflammation. Tumor metastasis requires migration of malignant cells from the original tumor to other sites within the body. This process requires a number of chemotactic signals that allow the cells to migrate to nearby vessels, enter the vasculature, and extravasate at distal sites. Similarly, inflammation involves recruitment of leukocytes and other inflammatory cells from the vasculature to injured tissue and numerous changes within the tissue such as epithelial proliferation, extracellular matrix deposition, and functional alterations in both the invading and host cells. Several studies over the last decade in tumor cell lines have demonstrated that constitutive migration of many cancer cells is dependent upon β-arrestin (Ge et al. 2004; Zabel et al. 2009). Likewise, studies have demonstrated that certain inflammatory processes are impaired in the absence of β-arrestin-2. Furthermore, β-arrestin-dependent regulation of actin-cytoskeletal proteins and signaling pathways that affect cell migration has been demonstrated in human cancer cell lines and leukocytes (Zoudilova et al. 2007, 2010).

Recent in vivo studies have shed more light on the multiple mechanisms by which β-arrestins can promote tumor cell migration and metastasis. Interestingly, these studies suggest that both tumor cell and host β-arrestin play important roles in this process and that β-arrestins are pro-metastatic in some scenarios and anti-metastatic in others. In many cases, β-arrestin complexes described in the previous sections play a major role in the metastatic progression of cancers in vivo. CXCR4, which we discussed earlier in the chapter, is upregulated in numerous malignant cancers and CXCL12 is expressed in many of the tissues to which tumor cells commonly metastasize. While CXCL12 monomers promote metastasis, CXCL12 dimers have tumor suppressor functions, effectively inhibiting cell migration through a β-arrestin-independent pathway (Drury et al. 2011). Thus, development of biased ligands that act like CXCL12 dimers may have therapeutic value for the treatment of some cancers.

Several reports have also indicated that β-arrestins are required for the recruitment of immune cells to the airways during asthma (see chapter "GPCRs and Arrestins in Airways: Implications for Asthma"). Influx of leukocytes to the lungs was not only decreased in β-arrestin-2 knockout mice but in wild type mice receiving knockout bone marrow. Thus, the role of β-arrestins in asthma appears to be mediating the chemotaxis of invading inflammatory cells (Walker et al. 2003; Hollingsworth et al. 2010; Nichols et al. 2012). In another inflammatory process, pulmonary fibrosis, a major contributing factor is the uncontrolled invasion of the extracellular matrix by fibroblasts and the secretion of matrix components. In a mouse model of pulmonary fibrosis, β-arrestin-1 or β-arrestin-2 knockout mice were protected from excessive matrix deposition, resulting in protected lung function and increased survival. Knockdown of either β-arrestin in fibroblasts from patients with pulmonary fibrosis inhibited their migration and invasive behavior (Lovgren et al. 2011). Thus, in this context β-arrestins were negatively regulating an inflammatory process. However, a resolution to these disparate findings is found when one looks at the underlying role of chemotaxis in both processes. β-arrestin-dependent chemotaxis in vivo contributes to recruitment of inflammatory cells, which can result in chronic inflammation. In contrast, in the sepsis studies in which β-arrestin-2 appears to negatively regulate tissue damage and mortality resulting from sepsis (Fan et al. 2010), the focus was not on β-arrestin-stimulated chemotaxis, but rather production of inflammatory mediators important for resolving bacterial infections. Release of these cytokines may be dependent upon the G-protein signaling arm of chemokine receptors and thus inhibited by β-arrestins. It is important to bear in mind that the role of β-arrestins in processes requiring chemotaxis in vivo is far more complicated than their role in chemotaxis in vitro. Depending on the physiological scenario and the receptor being activated, the classical role of β-arrestins as terminators of G-protein signaling dominate or their role as facilitators of chemotaxis may dominate.

5 Concluding Remarks

Although it has been over a decade since the demonstration that β-arrestins are important for chemotaxis downstream of numerous GPCRs, much remains to be elucidated regarding the underlying molecular mechanisms. Clearly receptor turnover and ligand degradation via β-arrestin-dependent endocytosis is important, but evidence suggests that the story is far more complicated than this. β-Arrestins are capable of regulating, both spatially and temporally, a wide array of cellular activities essential for cell migration. Since the spatiotemporal regulation of signals generated during cell migration must be tightly controlled, it stands to reason that the role of β-arrestins as signaling scaffolds is equally important in cell migration as is their role in gradient sensing. This chapter summarizes the experimental evidence supporting multiple roles for β-arrestins in chemotaxis. Given the plethora of biological responses—from inflammation to cancer—that are controlled by β-arrestin-dependent regulation of actin assembly and chemotaxis, it is clear that much more needs to be learned regarding the molecular mechanisms underlying this process.

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Arrestins in Host–Pathogen Interactions

Stefano Marullo and Mathieu Coureuil

Contents

Abstract In the context of host–pathogen interaction, host cell receptors and signaling pathways are essential for both invading pathogens, which exploit them for their own profit, and the defending organism, which activates early mechanism of defense, known as innate immunity, to block the aggression. Because of their central role as scaffolding proteins downstream of activated receptors, β-arrestins are involved in multiple signaling pathways activated in host cells by pathogens.

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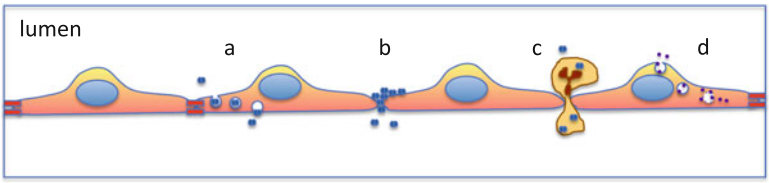

Fig. 1 Cellular pathways through which microorganisms cross the endothelia or penetrate into the cytoplasm. Extracellular pathogens may cross the endothelial monolayer through transcellular penetration following endocytosis (a) via paracellular entry after disruption of endothelial cell tight junctions (b) or by transmigration with infected leukocytes (Trojan horse mechanism, c). Intracellular pathogens and toxins can also penetrate into the cytoplasm via endocytosis and subsequent crossing of endosomal membranes (d)

Some of these pathways participate in the innate immunity and the inflammatory response. Other β-arrestin-dependent pathways are actually hijacked by microbes and toxins to penetrate into host cells and spread in the organism.

Keywords Innate immune response • Toll-Like Receptors • NF-κB • Sepsis • Pneumococcus • Meningococcus • Blood–brain barrier • Filoviridae • HTLV-1 • Anthrax

1 Introduction

Host–pathogen interactions are complex multifacet phenomena determining how host cells are colonized and how pathogens can disseminate (Fig. 1). Intracellular bacteria, viruses, and microbial toxins penetrate into host cell after crossing plasma membranes. They can then proliferate and/or spread to invade host tissues. Extracellular pathogens, instead, need to cross the mechanical barrier constituted by skin, airways, gut, urinary, or genital tract epithelia to diffuse to the bloodstream and colonize organs. Crossing the first layer of epithelial cells and then endothelia can be achieved by different mechanisms. Pathogens can pass through these barriers via endocytosis at their apical (for epithelia) or luminal (for endothelia) side and then be shuttled inside vesicles to the basolateral side, a phenomenon known as transcytosis. They can also disseminate through the intercellular space between two adjacent cells via the so-called paracellular route. Finally, they can first infect blood cells, which are physiologically capable of crossing epithelia and endothelia (by diapedesis), and carry the hidden pathogen to the other side of the barrier like a Trojan horse.

In all these cases, early steps of infection usually require pathogen adhesion to host cells via specific interaction with cell surface receptors. Then, pathogen

binding to host cell receptors induces signaling events leading to important changes in cell metabolism, shape, organization, and trafficking, which are exploited by the pathogen for proliferation and productive infection. On the other hand, pathogens must circumvent host cell responses, which induce signaling cascades leading to inflammation and other early mechanism of defense known as innate immunity. Thus, in the context of host–pathogen interaction, host cell receptors and signaling pathways are essential for both pathogens and the defending organism.

2 Beta Arrestins Are Multitask Proteins, Which Regulate Cell Surface Receptors and Orchestrate Signaling Pathways in Time and Space

Beta arrestins 1 and 2 (βarr1 and βarr2, also called arrestin-2 and -3) are the ubiquitous isoforms related to visual arrestin-1, which in the retinal tissue is responsible for the "arrest" of rhodopsin activation. It is not surprising therefore, that βarrs were originally identified as negative regulators of G protein-coupled receptor (GPCR) function in non-retinal tissues, because their binding promotes GPCR desensitization (Lohse et al. 1990) (see chapter "Arrestin Interactions with G Protein-Coupled Receptors"). Indeed, the translocation of cytoplasmic βarrs to activated and phosphorylated receptors uncouples GPCRs from downstream G protein-dependent signaling pathways. βarrs were subsequently shown to play a role as adaptor proteins connecting activated and phosphorylated GPCRs to AP2 and clathrin, two components of the endocytic machinery (Goodman et al. 1996; Laporte et al. 1999) (see chapter "β-Arrestins and G Protein-Coupled Receptor Trafficking"). Thanks to this molecular bridge; GPCRs are recruited in clathrin-coated pits and subsequently internalized into endosomes. Successive investigations extended the spectrum of the roles of βarrs in receptor trafficking. Indeed, βarrs promote the recruitment of ubiquitin ligases and thus participate in the agonist-induced ubiquitylation of receptors, which impacts on their subcellular localization and stability (see chapter "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications"). Ubiquitination, in addition to its well-known function in soluble protein proteasomal degradation, serves as a signal to recruit ubiquitin-binding domain-containing proteins, for specific biological functions, such as endocytosis or sorting to lysosomes (Chen and Sun 2009). Mdm2 was the first E3 ligase recognized as a βarr partner; by ubiquitilating βarrs, it provides a signal necessary for the internalization of βarr-bound GPCRs (Shenoy et al. 2001). Other E3 ligases, such as NEDD4 or AIP4, were instead reported to provide a lysosome-sorting signal to internalized receptors (Shenoy et al. 2008; Marchese et al. 2003). βarrs were also found to participate in internalization or ubiquitylation (or both) of many non-GPCR receptors or plasma membrane proteins: the type III transforming growth factor-β receptor, the insulin-like growth factor I receptor, voltage-

dependent calcium channels, the Na(+)/H(+) exchangers NHE1 and NHE5, the vascular endothelial (VE) cadherin, and Notch [reviewed in Shukla et al. (2011)]. Interestingly, recent studies have identified a larger and more ancient family of arrestin-fold proteins that display some structural similarity with βarrs and share their trafficking and downregulating functions. This family of "α-arrestins" is conserved in eukaryotes (Alvarez 2008) and comprises ARRDC (Arrestin domain-containing) proteins (Nabhan et al. 2010; Patwari et al. 2011) in mice and humans and ARTs (arrestin-related trafficking adaptors) in yeast (Lin et al. 2008).

In addition to their role in receptor desensitization and trafficking, βarrs have a function of signaling adaptors and scaffolds (see chapters "Arrestin-Dependent Activation of ERK and Src Family Kinases," "Arrestin-Dependent Activation of JNK Family Kinases," and "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences"). Assembling signaling proteins into molecular hubs (or signalosomes) constructed around scaffolding proteins is a common mechanism used by all cells to correctly deliver specific signals in space and time (Good et al. 2011). Since the first description of the Ste5 scaffold of the MAP kinase cascade in yeast (Choi et al. 1994), an increasing number of protein scaffolds have been identified, based on their ability to bind multiple signaling partners via direct protein–protein interactions, due to their high content of modular protein-binding domains (Zeke et al. 2009). After the pioneering reports describing the role of βarrs in organizing the oriented activation of MAP kinases in the cytoplasm (McDonald et al. 2000; Luttrell et al. 2001), many other effector pathways orchestrated by βarrs have been characterized [reviewed in Shenoy and Lefkowitz (2005), Lefkowitz et al. (2006), Kovacs et al. (2009), Luttrell and Gesty-Palmer (2010)] illustrating the prominent role of βarrs in the control of cell signaling.

3 βarrs in the Host Cell Response to Pathogens

Because of their central role as scaffolding proteins downstream of activated receptors, βarrs are involved in multiple pathways activated in host cells by pathogens. The important phenomenon of βarr-dependent regulation of cell motility and chemotaxis, via the control of actin polymerization and cytoskeletal rearrangements, is described in another chapter of this book (see chapter "Molecular Mechanisms Underlying Beta-Arrestin-Dependent Chemotaxis and Actin-Cytoskeletal Reorganization"). Here we will summarize the principal established roles of βarrs in innate immunity, inflammatory response, and apoptosis (Table 1).

Table 1 βarrestins in the host cell response to pathogens

βarr target(s)	βarr isoform	Activation (A) or Inhibition (I)	Signaling effect	Reference
Hck/c-Fgr	Both	A	Contribute to granule release	Barlic et al. (2000)
IkBα	βarr2	I (NF-κB pathway)	Prevents the phosphorylation and degradation of IκBα	Gao et al. (2004)
TRAF6	Both	I (NF-κB pathway)	Prevent TRAF6 auto-ubiquitination	Wang et al. (2006)
ND	βarr2	A	Mediates LPS-induced ERK 1/2 activation	Fan et al. (2007)
ND	both	I	Mediate LPS-induced NFκB	Fan et al. (2007)
ND	βarr1	A	Adenovirus–vector-induced innate immune responses	Seregin et al. (2010)
ND	βarr2	I	Adenovirus–vector-induced innate immune responses	Seregin et al. (2010)
P105	βarr1	I	Modulates the MAP kinase arm of TLR4 signaling	Parameswaran et al. (2006)
GSK-3b	βarr2	I (apoptosis)	Stabilization of phospho-GSK-3b (inactive form of GSK-3b)	Li et al. (2010)
KIR2DL1	βarr2	I (NK response)	Recruitment of the tyrosine phosphatases SHP-1,2	Yu et al. (2008)

3.1 βarr Involvement in Leukocyte Degranulation

The first evidence for a βarr involvement in innate immunity came from studies on chemoattractant-stimulated granule release in leukocytes (Barlic et al. 2000). Leukocyte granules contain several enzymes and nonenzymatic compounds that participate in bactericidal activity. The release of these granules is controlled by the activation of Fc receptors or GPCRs for chemoattractants. Interleukin 8 (IL-8) activation of the chemokine receptor CXCR1 was found to stimulate rapid formation of βarr complexes with the Src-family tyrosine kinases Hck or c-Fgr. Hck association with βarrs activates the kinase and allows its targeting to granules. In case of expression of dominant-negative βarr mutant with altered polyproline-rich region (known to be critical for the interaction with the c-Src tyrosine kinase), granulocytes fail to release granules or activate tyrosine kinases in response to IL-8 stimulation. Thus, in this pathophysiological context, βarrs are important signaling molecules in the innate immune response.

3.2 βarr Regulation of Toll-Like Receptor Signaling

Pathogen-associated molecular patterns (PAMPs) (Janeway 1989) such as flagellin, the lipopolysaccharide (LPS), or the peptidoglycan of bacterial cell wall are recognized by specific host receptors known as pattern-recognition receptors (PRRs). During infection, PAMPs-mediated activation of PRRs initiates inflammatory reactions, which constitute the first line of defense and prepare the establishment of adaptive immune responses. Several classes of PRRs have been described, among which the Toll-like receptors (TLRs) are key initiators of the innate immune response (Medzhitov et al. 1997). Some TLRs (1, 2, 4, 5, and 6) operate primarily at the plasma membrane, whereas other TLRs, mostly involved in the recognition of nucleic acids, are localized to late endosomes and lysosomes. Signal transduction mechanisms of TLRs are similar to those elicited by some interleukin receptors. TLRs contain a Toll interleukin-1 receptor homology (TIR) domain (O'Neill and Bowie 2007), which engages cytoplasmic TIR-domain-containing adaptors such as the myeloid differentiation primary response gene 88 (MyD88) or the TIR domain containing adaptor protein (TIRAP). These adaptors recruit members of the IRAK (IL-1 receptor-associated kinase) family of serine–threonine kinases that induce inflammatory cytokine expression. MyD88 and TIRAP promote the expression of nuclear factor NF-kB-dependent cytokines via the activation of NFκB and of mitogen-activated protein kinases, whereas other adaptors induce the expression of type I interferons (IFNs).

The first indication that βarrs can modulate TLR signaling was based on the observation that βarr2 (not βarr1) directly interacts with IkBα (Gao et al. 2004). The protein kinase IKK, activated by phosphorylation downstream of stimulated TLRs or the TNF receptor, phosphorylates IκBα that normally binds to the transcription factor NF-κB and inhibits its nuclear translocation. Once phosphorylated, IκBα is ubiquitinated and targeted for degradation by the proteasome, releasing NF-κB. NF-κB-containing heterodimers then translocate into the nucleus and mediate the transcription of a vast array of proteins involved in immune and inflammatory responses. Interaction with βarr2 prevents the phosphorylation and degradation of IκBα. Interestingly, GPCR stimulation can enhance βarr2–IκBα interaction and consecutive stabilization of IκBα, leading to the inhibition of the NF-κB pathway. Supporting the hypothesis that βarrs are negative regulators of the innate immune activation via TLRs, it was reported that both βarr isoforms interact with TRAF6 preventing its auto-ubiquitination and subsequent activation of NF-κB (Wang et al. 2006). TRAF6 is a ring domain E3 ubiquitin ligase that it is involved in the activation of IKK downstream of TLRs and IL-1 receptor; it interacts with βarrs upon stimulation by IL1-β or gram-negative bacteria lipopolysaccharide. Consistently, endotoxin-treated βarr2-deficient mice had higher expression of pro-inflammatory cytokines and were more susceptible to endotoxin shock than controls. A subsequent study comparing wild type and βarr2-deficient mice confirmed that βarr2 is a negative regulator of the inflammatory response in polymicrobial sepsis (Fan et al. 2010). However, the existence of different

functional outputs in mouse models investigated with diverse experimental approaches (Porter et al. 2010), indicate a more complex regulation of TLRs response by βarrs. Part of the explanation might be that βarr1 and βarr2 differentially regulate TLR signaling and pro-inflammatory gene expression. For example, one study reported that both βarrs negatively regulate LPS-induced NFκB, whereas only βarr2 mediates LPS-induced ERK 1/2 activation (Fan et al. 2007). Also, in a report examining adenovirus–vector-induced innate immune responses and involving TLR-dependent pathways, βarr1 was found to be a positive regulator and βarr2 a negative regulator (Seregin et al. 2010). The functional output of the specific involvement of each βarr isoform might also vary in different cell types. In macrophages, both βarr1 and the G protein receptor kinase GRK5 inhibit LPS-dependent signaling of the TLR4. More specifically, βarr1 (not βarr2) modulates the MAP kinase arm of TLR4 signaling by interacting with NFκB1 p105, which is the precursor of NFκB1 p50 and a cytoplasmic inhibitor of NF-κB: p105 functions as an IκB and retains associated p50 in the cytoplasm. As described in fibroblasts for βarr2, which directly interacts with IkBα preventing its phosphorylation and degradation (Gao et al. 2004), βarr1 stabilizes p105. Knockdown of βarr1 leads to enhanced LPS-induced phosphorylation and degradation of p105, enhanced MAP3K release, and enhanced MAP2K phosphorylation (Parameswaran et al. 2006).

In addition to its role in the inflammatory response via the NFκB and the MAP kinase pathways, TLR4 activation can promote apoptosis under certain conditions and in some cell types (Gay and Gangloff 2007). A recent study identified the glycogen synthase kinase-3b (GSK-3b) as an intermediate for TL4-mediated apoptosis (Li et al. 2010). Interestingly, the apoptotic cascade was attenuated by βarr2, likely via the stabilization of phospho-GSK-3b, an inactive form of GSK-3b.

3.3 βarr Regulation of Natural Killer Cells

Natural killer (NK) cells are critical components of the innate immune system that recognize and kill tumor or virus-infected target cells. These cells express at their surface two sets of receptors. Activating receptors that are involved in the killing activity of NK cells, whereas inhibitory receptors contribute to tolerance to normal healthy cells. The association of the inhibitory receptor KIR2DL1 with βarr2 was reported to induce the recruitment of the tyrosine phosphatases SHP-1 and SHP-2 to KIR2DL1, contributing to the inhibitory signaling. Cytotoxicity of NK cells is consequently higher in βarr2-deficient mice and inhibited in animals overexpressing βarr2. The inhibitory effect of βarr2 is functionally relevant in vivo, as shown by decreased NK cell-dependent susceptibility to cytomegalovirus infection in βarr2-deficient mice (Yu et al. 2008).

Table 2 βarr-dependent pathways hijacked by microbes and toxins

βarr target(s)	Pathogen	Arrestin family member	Functional effects	Reference
PAF receptor	*Streptococcus pneumoniae*	Both βarrs	Activation of ERK, transcytosis	Radin et al. (2005)
β2-adrenoceptor, Src, p120, VE-cadherin	*Neisseria meningitides* *Neisseria gonorrheae*	Both βarrs	Stabilize adhesion to endothelial cells; open anatomical gaps between adjacent endothelial cells	Coureuil et al. (2010)
TIM-1 receptor	Marburg virus	βarr1	AP1- and clathrin-dependent endosomal sorting	Bhattacharyya et al. (2011)
HECT ubiquitin ligases	HTLV-1	ARRDC (Arrestin domain-containing)	Promote ESCRT-III recruitment; viral budding	Rauch and Martin-Serrano (2011)
CMG2/TEM8 receptors	*Bacillus anthracis* toxin	βarr1	Receptor ubiquitination; recruitment of AP-1 adaptin and clathrin; endocytosis	Abrami et al. (2010a)

4 Receptors and Signaling Pathways Involving βarrs That Are Hijacked by Microbes and Toxins to Penetrate into Host Cells or Spread (Table 2)

4.1 Bacteria

Streptococcus pneumoniae (pneumococcus), a gram-positive pathogen causing pneumonia, sepsis, and meningitis, is the first reported example of bacteria exploiting βarrs for tissue invasion. Pneumococci translocate across human endothelial cells through vesicular structures without intracellular multiplication (transcytosis). Early studies identified the receptor for platelet-activating factor (PAF) as the pneumococcus adhesion receptor in both epithelial and endothelial cells (Cundell et al. 1995; Ring et al. 1998). Pneumococcus binding to PAF receptors induces βarr translocation and endocytosis of pathogen–receptor complexes. Cytoplasmic activation of ERK, presumably mediated by βarrs, is required for pneumococcal endocytosis (Radin et al. 2005). Interestingly, instead of being directed to lysosomes or recycled to the cell surface as agonist-bound receptors, a significant proportion of bacteria–PAF receptor complexes are

diverted to basolateral membranes, this proportion being enhanced by βarr overexpression. Thus, pneumococci subvert the βarr-dependent trafficking machinery of PAF receptors to drive pathogen-containing vacuoles away from lysosomes and across endothelial cell barriers (Radin et al. 2005).

N. meningitidis (meningococcus) is a Gram-negative diplococcus causing cerebrospinal meningitis and "purpura fulminans," a severe disseminated form of infection with peripheral vascular leakage, ischemic tissue damage, and septic shock. The ability of meningococci to interact with endothelial cells is essential in meningococcal pathogenesis (Coureuil et al. 2012). After initial attachment, mediated by a still unidentified receptor, bacteria have the ability to resist blood flow, to multiply and form microcolonies on the apical surface of endothelial cells. The stabilization of bacterial colonies depends on the formation of host cell protrusions, which occur in response to signaling cascades elicited by the pathogen in the endothelial cells. In addition, bacterial-induced signaling eventually results in the opening of intercellular junctions with subsequent meningeal colonization via the paracellular route (Coureuil et al. 2009). It has been established that signaling in host cells is provoked by polymeric filaments found on many Gram-negative bacteria, known as type IV pili, which correspond to the multimeric assembly of various pilin subunits (Miller et al. 2012). Recently, it was reported that *N. meningitidis* pilins allosterically stimulate a biased β2-adrenoceptor-βarr signaling pathway in endothelial cells, which ultimately traps βarrs and their interacting partners, such as the Src tyrosine kinase and junctional proteins VE-cadherin and p120, under bacterial colonies (Coureuil et al. 2010). The cytoskeletal reorganization mediated by βarr-activated Src stabilizes bacterial adhesion to endothelial cells under permanent flow, whereas βarr-dependent delocalization of junctional proteins results in anatomical gaps between adjacent endothelial cells, which are used by bacteria to penetrate into tissues. The bacterial ligand, which activates the β2-adrenoceptor by interacting with the receptor N-terminal region, corresponds to two particular components of the pili, namely the pilins PilE and PilV.

N. gonorrheae (gonococcus) a close relative of meningococcus, which most often causes isolated infection of the genitourinary tract but can, in rare cases, spread into the bloodstream and colonize meninges (Martín et al. 2008), elicits similar signaling events as *N. meningitidis* in endothelial cells (Coureuil et al. 2010). In addition, many other bacteria take advantage of host cell signaling pathways involving Src activation and its substrate cortactin to invade tissues, as in the case of *Neisseria* species. Although not investigated yet, βarrs might well participate in the signaling pathways induced by these other pathogens.

4.2 Viruses

Marburg virus (MARV) and Ebola virus (EBOV), two members of the Filoviridae family, are the causative agents of a deadly infection, known as viral hemorrhagic fever (Schnittler and Feldmann 2003). Although several monocyte, macrophage, dendritic, and endothelial cell surface proteins have been implicated in filovirus entry, a common receptor, the T-cell immunoglobulin and mucin domain 1 (TIM-1), was reported for both Ebola and Marburg viruses (Kondratowicz et al. 2011). Following viral glycoprotein (GP)-dependent receptor binding, filoviruses are internalized by clathrin-mediated endocytosis. The cellular endocytic machinery sorts internalized viruses to an acidic endosomal compartment, which is the site of virus–cell membrane fusion. A recent report examined the specific requirements for different components of the clathrin endocytic machinery in Ebola GP versus Marburg GP pseudovirion entry (Bhattacharyya et al. 2011). Whereas Ebola GP pseudovirions specifically required the adaptor proteins Eps15 and AP-2 to be connected to clathrin, Marburg GP pseudovirions specifically needed βarr1 and the adaptor protein AP-1 instead of AP-2. Knocking down βarr1 significantly delayed virus fusion with no evident virus-binding defect.

The endosomal sorting complex required for transport (ESCRT) machinery comprises multiprotein complexes (ESCRT 0–III) that cooperate in a sequential and a coordinated manner to target ubiquitinated membrane cargo into vesicles that bud into late endosomes to form multivesicular bodies (MVBs) (Hurley and Emr 2006). In particular, internalized cell surface receptors that are programmed for lysosomal degradation are delivered to MVBs via this machinery. Internalized ubiquitinated receptors in the endosomes are initially recognized by ESCRT-0, which subsequently recruits ESCRT-I to endosomal membranes, followed by recruitment of ESCRT II and III. The process terminates with receptor sorting into budding intra-endosomal vesicles (Raiborg and Stenmark 2009). For some receptors, such as the chemokine receptor CXCR4, βarr1 connects the ubiquitinated receptor with ESCRT-0 and regulates the amount of CXCR4 that is degraded (Malik and Marchese 2010). ESCRT machinery also plays a key role in the budding of many enveloped viruses, including HIV-1 and other retroviruses. Recently, it was reported that ARRDC (Arrestin domain-containing) proteins are involved in budding of murine leukemia virus or human T-cell leukemia virus type 1 by interacting with HECT ubiquitin ligases and promoting ESCRT-III recruitment (Rauch and Martin-Serrano 2011).

4.3 Toxins

Bacillus anthracis, the bacterium responsible for the anthrax disease produces the anthrax toxin, which is composed of three independent polypeptide chains. Two proteins have an enzymatic activity (calmodulin-dependent adenylate cyclase and

metalloprotease, cleaving the MAP kinase kinase, respectively) the third one being required for the translocation of the two enzymes into the cytoplasm where their activity produces the toxic effects (Young and Collier 2007). The protein involved in toxin translocation, known as the protective antigen (PA), interacts with the target cells. It is processed by host cell proteases, such as furin, leading to the formation of a 63 kDa fragment that heptamerizes into a ring like structure. The complex between the heptamer and the two enzymes is internalized into endosomes where the heptamer forms a pore allowing the partially unfolded/activated enzymes to cross the endosomal membranes and reach the cytosol (Collier 2009). Heptamerization of the 63 kDa PA fragment leads to the activation of src-like kinases (Abrami et al. 2010b), which phosphorylate the cytoplasmic tail of capillary morphogenesis 2 (CMG2) a type-I membrane protein that serves as toxin receptor. Toxin receptors (CMG2 and also the tumor endothelial marker 8 TEM8) are ubiquitinated, via a process that requires βarr1. Receptor modification finally allows the recruitment of AP-1 adaptin and clathrin, leading to their internalization via clathrin-coated pits (Abrami et al. 2010a).

5 Conclusions and Perspectives

Facing the vast array of pathogen ligands and of potential host cell receptors that have been selected by pathogens to penetrate into host cells or to cross epithelial and endothelial barriers, the number of cellular pathways hijacked by pathogens or employed by host cells as primary defense line are relatively limited. Moreover, a restricted set of proteins such as kinases (Src family, MAP kinases), proteins involved in endocytosis and sorting, junctional proteins, signaling adaptors at the cross-road of various pathways downstream of TLRs or cytokine receptors, is constantly involved in these processes, whatever the type of the pathogen. Most of these proteins appear as direct or indirect interactors of arrestins, suggesting that our current knowledge of the role of arrestins in host–pathogen interactions only represents the tip of the iceberg.

A remarkable feature of βarrs, is the large number of cellular proteins they interact with, contrasting with limited amino-acid residues and areas of contact involved in individual interactions (Gurevich and Gurevich 2012). This feature might be exploited to develop very specific molecules capable of targeting signaling pathways at the appropriate level and with exquisite precision for therapeutic purposes. In this context, host–pathogen interactions appear a particularly interesting area of investigation.

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Arrestin Regulation of Small GTPases

Ryan T. Cameron and George S. Baillie

Contents

Abstract The regulation of small GTPases by arrestins is a relatively new way by which arrestin can exert influence over cell signalling cascades, hence, molecular interactions and specific binding partners are still being discovered. A pathway showcasing the regulation of GTPase activity by β-arrestin was first elucidated in 2001. Since this original study, growing evidence has emerged for arrestin modulation of GTPase activity through direct interactions and also via the scaffolding of GTPase regulatory proteins. Given the importance of small GTPases in a variety of essential cellular functions, pharmacological manipulation of this pathway may represent an area with therapeutic potential, particularly with respect to cancer pathology and cardiac hypertrophy.

Keywords Small GTPase • Guanine nucleotide exchange factor (GEF) • GTPase-activating protein (GAP) • β-Arrestin • ARHGAP21

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1 What Are Small GTPases?

Small GTPases are monomeric GTP-binding proteins with molecular weights ranging from 20 to 40 kDa. They cycle between biologically active GTP- and inactive GDP-bound conformations. The exchange of GDP for GTP is mediated by a guanine nucleotide exchange factor (GEF), which in turn promotes interaction with downstream effectors. The intrinsic GTPase activity of small GTPases is relatively slow and is enhanced through GTPase-activating proteins (GAPs). These regulators of GTPase activity either can be specific to individual GTPases or can have subfamily specificity (Takai et al. 2001). The structural properties of GTPases are highly conserved and serve as molecular switches, where exchange of GDP for GTP is a versatile way to alter affinities for various effector proteins to allow for the conversion of extracellular signals into a wide variety of cellular outcomes (Bourne et al. 1991).

GTPases were first discovered in the 1980s as the oncogenes of rat sarcoma viruses and mutated orthologs were subsequently found in several human carcinomas. These mutated forms of the GTPases, termed Ras (Rat sarcoma), were shown to stimulate proliferation and transformation of cultured cell lines (Chien et al. 1979; Parada et al. 1982; Santos et al. 1982). There have now been more than 100 small GTPases identified in eukaryotes, which represent the Ras superfamily of proteins. There are several structurally distinct members of the family, which can be classified into their respective subfamilies that are characterised by differing functional roles: the Ras subfamily members are involved mainly in gene regulation, the Rho subfamily which includes widely studied Rho/Rac/Cdc42 proteins regulates cytoskeleton reorganisation and gene expression, the Rab and Arf subfamilies are involved in intracellular vesicle trafficking, the Ran subfamily regulates nuclear trafficking and coordinates cell cycle events, and finally the more recently added subfamily, the Miro GTPases, regulates mitochondrial dynamics (Takai et al. 2001; Reis et al. 2009).

2 Arrestin Regulation of Small GTPases

β-Arrestins have a long-established role in regulating G protein-coupled receptor endocytosis and desensitisation (see Chaps. 2, 4, 7 and 9). However, a novel mechanism for β-arrestin-mediated regulation of GPCR internalisation was discovered by Claing et al. (2001). They showed that the activation of the small GTPase ADP-ribosylation factor 6 (ARF6) was essential to mediate receptor endocytosis. By demonstrating that mutants of ARF6, which were unable to bind or hydrolyse GTP, caused attenuation of β_2-adrenergic receptor (β_2-AR) internalisation following isoproterenol stimulation, a novel function of arrestin was unearthed. Follow-up work highlighted the fact that the expression of a GEF for ARF6 (ARF nucleotide binding site opener (ARNO)) activated ARF6 and enhanced receptor endocytosis,

while the ARF-specific GAP, GIT1, inhibited internalisation of β2-adrenergic receptor. Given the role that β-arrestins play in receptor endocytosis, these authors hypothesised that β-arrestins may be regulating ARF6 function. Utilising immunoprecipitation techniques, they proved that both β-arrestin1 and 2 were associated with ARF6 and that the interaction was enhanced following isoproterenol stimulation. Importantly, it was the GDP-bound form of ARF6, which co-precipitated with β-arrestin1, and the addition of GTPγS abolished this interaction, typifying the molecular switching of the GTPase interactome following activation. Therefore, once ARF6 becomes activated, it disassociates from β-arrestin and is able to promote endocytosis. As well as establishing for the first time a direct interaction of β-arrestin with a small GTPase, Claing et al. found that β-arrestin also interacted with the ARF6 GEF, ARNO. This interaction occurred under both basal and stimulated conditions, and ARNO was found in a complex containing both β-arrestin1 and ARF6. Furthermore, by utilising an in vitro GTPγS loading assay, they demonstrated that β-arrestin1 significantly potentiated the activation of ARF6 by ARNO, suggesting that β-arrestins play a direct role in regulating GTPase activity, which in this setting, would directly promote endocytosis of the β2-adrenergic receptor (Claing et al. 2001).

A second study showing the regulation of GTPase activity, used a yeast two-hybrid screen of human mammary gland and brain cDNA to uncover the role of arrestin. In order to identify signalling proteins, which may interact with β-arrestins, the authors used the N terminus of β-arrestin2 as the bait protein and isolated six clones which encoded two overlapping domains of Ral-GDS. Ral-GDS is a GEF for Ral (a GTPase) involved in chemotactic migration. Further studies carried out in HEK 293 cells demonstrated that a GFP-tagged version of Ral-GDS co-immunoprecipitated with both β-arrestin1 and 2. Ral had previously been shown to be activated by the formyl-Met–Leu–Phe (fMLP) receptor, a GPCR which detects a bacterial tripeptide and stimulates a variety of functional responses in immune cells such as neutrophils, in a Ras-dependent and Ras-independent manner. A GFP-tagged fMLP receptor was utilised to examine membrane ruffling as an indicator of cytoskeleton reorganisation. Membrane ruffling could be blocked following fMLP stimulation by expressing the N terminus of β-arrestin1. However, the expression of the C-terminal domain of β-arrestin1 had no effect on membrane ruffling. Further studies showed that fMLP-induced membrane ruffling could also be blocked via expression of dominant-negative RalA, while constitutively active RalA promoted membrane ruffling in the absence of fMLP stimulation. A constitutively active Ras mutant did not promote membrane ruffling, demonstrating that Ras-independent β-arrestin1 activation of Ral-GDS/Ral signalling pathway is required for cytoskeletal reorganisation in this instance.

A GTPγS uptake assay was utilised to determine the effect that the β-arrestin–Ral–GDS interaction would have on RalA activation. Overexpression of β-arrestin did not alter fMLP-stimulated RalA GTPγS binding; however, expression of the N-terminal domain of β-arrestin1 or a Ral–GDS clone 284, which encompassed a domain with no known GEF function (amino acids 616–768), completely abolished GTPγS binding. Expression of either the N-terminal domain

of β-arrestin1 or Ral–GDS clone 284 blocked the co-immunoprecipitation of Ral–GDS with β-arrestin1 or of β-arrestin1 with Ral–GDS. Using purified RalA, β-arrestin1, and Ral–GDS, it was shown that β-arrestin1 was able to attenuate Ral–GDS-mediated activation of RalA. This suggested that β-arrestin1 maintains Ral–GDS in an inactive state prior to fMLP receptor activation. The β-arrestin1/Ral–GDS complex was disrupted following 15 s of fMLP stimulation but was shown to re-associate after 5 min. The re-association correlated with an end of fMLP-induced membrane ruffling. Within this window, Ral–GDS was shown to be redistributed from the cytosol to the plasma membrane and was enriched in membrane protrusions. Translocation of Ral–GDS to the plasma membrane was blocked by the expression of the N-terminal half of β-arrestin1 and Ral–GDS clone 284. Taken together, these data implicate β-arrestin1 as a key regulator of fMLP receptor-mediated activation of RalA, and this, in turn, modulates cytoskeletal dynamics and promotes a number of cellular responses essential for immune cell functions such as chemotaxis and granule release (Bhattacharya et al. 2002).

The importance of β-arrestin1 regulation of the Ral–GDS/Ral signalling pathway has also been noted in several human cancer cells. In a study of breast cancer cells, HS578T and MDA-MB-231, and a melanoma cell line, MDA-MB-435, up-regulation of β-arrestin1 mRNA was evident when a comparison with a non-tumorigenic cell line (MCF-10A cells) was undertaken. It was also shown that lysophosphatidic acid receptors 1 and 2 (LPA_{1-2}), which play a role in cancer progression, along with β-arrestin2 and Ral GTPase mRNA levels were significantly increased in advanced stages of breast cancer. As β-arrestins had been shown previously to modulate Ral GTPase activity via Ral–GDS, stable cell lines expressing either a dominant-negative mutant of Ral–GDS or the sequence containing the minimum binding site of Ral–GDS required to bind β-arrestin ($Ral–GDS^{616–768}$) were engineered. The dominant-negative mutant of Ral–GDS inhibited migration of MDA-MB-231 cells towards LPA, and more intriguingly, the expression of the $Ral–GDS^{616–768}$ peptide, in conjunction with, significant inhibition of migration, also resulted in the formation of spherical colonies typical of the non-tumorigenic MCF-10A cells (Li et al. 2009a, b).

To further validate the role of β-arrestins in mediating Ral-mediated breast cancer migration and invasion, short hairpin RNA was used to knockdown either β-arrestin1 or 2. Silencing of either arrestin isoform resulted in significant reduction in LPA-induced migration; however, there was no synergistic effect when both were knocked down simultaneously. The data discussed above, clearly indicate that LPA-mediated activation of Ral is β-arrestin dependent in breast cancer cells (Li et al. 2009a, b).

Work on the role of arrestin in RhoA-mediated signalling, found that β-arrestin1 is required to activate RhoA after the stimulation of the Ang II type 1A receptor ($AT_{1A}R$). Ang II stimulation of $AT_{1A}R$ has been implicated in cardiac myocyte remodelling and plays a role in the development of cardiac hypertrophy (Aoki et al. 1998). Previous reports had noted that Rho activation is mediated through the heterotrimeric G proteins $G_{\alpha q/11}$, $G_{\alpha 12}$ and $G_{\alpha 13}$. The $AT_{1A}R$ is known to signal mainly through $G_{\alpha q/11}$ to activate downstream effector proteins. Studies using

HEK 293 cells stably expressing $AT_{1A}R$ demonstrated that both β-arrestin1 and $G_{\alpha q/11}$ were required for full activation of RhoA and subsequent stress fibre formation. Targeted RNAi knockdown of either β-arrestin1 or 2 revealed that a reduction in β-arrestin2 levels led to slight increases in Ang II-induced RhoA activation, although this was not statistically significant, whereas targeted knockdown of β-arrestin1 led to a 60 % reduction in RhoA activation. The conclusion from this work was that β-arrestin1 is an essential component in the Ang II-RhoA signalling pathway; however, the mechanism by which β-arrestin1 recruitment to the $AT_{1A}R$ potentiates RhoA activation was not elucidated at that time (Barnes et al. 2005).

The mechanism by which β-arrestin1 activates RhoA was only recently discovered by Anthony et al. (2011). Utilising a yeast 2-hybrid approach, these researchers used β-arrestin1 as bait to probe a human brain RP1 library. They found that ARHGAP21, a member of the Rho GAPs, interacted with β-arrestin1 within the C-terminal Rho-GAP domain. ARHGAP21 can locate to the Golgi through an interaction with ARF1, where it can modulate the activity of several GTPases including Cdc42, Rac1 and RhoA. The interaction of β-arrestin1 with this segment of ARHGAP21 was further verified using peptide array technology. Libraries of overlapping peptides based on the entire ARHGAP21 Rho-GAP domain were SPOT synthesised onto a cellulose membrane. This procedure permits the overlaying of recombinant, tagged proteins and allows rapid characterisation of protein–protein interactions using "far"-Western blotting techniques. This methodology highlighted that the β-arrestin interaction with ARHGAP21 was exclusive to β-arrestin1, as no interaction was detected using recombinant β-arrestin2. A sequential alanine-scanning array was undertaken where native residues of ARHGAP21 were substituted for alanine in order to gauge their importance in facilitating binding of β-arrestin1. Using this method, several glutamic acid residues were identified within the sequence of ARHGAP21 that were essential for the β-arrestin1 interaction. Subsequent co-immunoprecipitation techniques were used to verify that the ARHGAP21 Rho-GAP domain was essential for the interaction with β-arrestin1.

To assess the role of β-arrestin1/ARHGAP21 binding in stress fibre formation, Ang II-$AT_{1A}R$-induced changes in cell morphology due to activated RhoA were evaluated. Cell-permeable peptides based on the β-arrestin1 docking domain of ARHGAP21 were developed in order to disrupt cellular pools of the β-arrestin1/ARHGAP21 complex. Treatment with such peptides resulted in a significant reduction in RhoA activation and attenuated subsequent stress fibre formation following Ang II treatment. These data suggest that β-arrestin1 inhibits ARHGAP21 GAP activity by binding directly at its Rho-GAP domain. When this is disrupted, ARHGAP21 rapidly hydrolyses the GTP-bound RhoA, leading to inhibition of RhoA signalling (Anthony et al. 2011).

β-Arrestin-mediated regulation of RhoA activation has also been examined in protease-activated receptor-1 (PAR1) signalling in human endothelial cells. β-Arrestin recruitment and activation of dishevelled-2 (Dvl-2) were investigated utilising targeted siRNA knockdown. Differential effects of anticoagulant protease-

activated protein C (APC) and the coagulant protein thrombin, on the activation of PAR1 was reported. Expression of β-arrestin was essential for APC-activated PAR1 cytoprotection in human endothelial cells due to β-arrestin linking Dvl-2 to Rac1, a critical factor for Rac1 activation (Soh and Trejo 2011).

Another study involving β-arrestin1 has shown its involvement in the early activation of p38 MAPK by the β_2-AR (Gong et al. 2008). Given that β-arrestin1 had been shown to modulate signalling of small GTPases and several Rho family members had also been shown to regulate activity of p38 MAPK, this study strived to determine what role Rho GTPases were taking in this signalling pathway. Utilising several broad-spectrum and isoform-specific Rho inhibitors, it was demonstrated that targeted inhibition of Rac1 also resulted in attenuation of the early p38 MAPK activation. Additionally, following transfection of dominant-negative mutants of RhoA, Cdc42 or Rac1, only the expression of the dominant-negative Rac1 elicited a significant decrease in p38 MAPK signalling. Finally, Rac1 activation was only completely blocked following treatment with selective inhibitor NSC23766 or knockdown of β-arrestin1, thereby asserting that β-arrestin1 is an upstream regulator of Rac1 activity (Gong et al. 2008).

The role of β-arrestins in β-adrenergic receptor internalisation and desensitisation has been studied extensively and is described in Chaps. 2 and 9. However, a novel signalling event involving β-arrestin regulation of small GTPases and β-adrenergic receptor function has recently come to light, which further highlights the scaffolding role played by β-arrestin in order to sequester regulators of GTPase activity to the vicinity of their targets. Work on β-arrestin2 and its interaction with the GEF, Epac1, showed that this complex had direct effects of signals from β-ARs that were channelled via Epac1 effectors Rap1 and H-Ras. Bioluminescence resonance energy transfer (BRET) techniques were utilised in order to monitor the interaction between β-arrestin2 and Epac1 in HEK 293 cells. By co-expressing Renilla luciferase (Rluc)-β-arrestin2 and YFP-Epac1 constructs, BRET could be detected between the two fluorophores, suggesting that β-arrestin2 and Epac1 interacted constitutively. These data were supported by co-immunoprecipitation assays, which demonstrated that isoproterenol stimulation did modify the pre-existing interaction.

The identification of binding sites between β-arrestin2 and Epac1 was solved using peptide array technology. This approach identified a domain, which included the Epac1 Ras association motif. Subsequent experimentation evaluated the Epac1-mediated activation of H-Ras and whether there was a signalling input from β-arrestin2. Measurement of Epac1 recruitment to the plasma membrane (following isoproterenol stimulation in cells co-transfected with either Epac1 β-arrestin2 siRNA or Epac1 with control siRNA) found that β-arrestin2 knockdown resulted in a 50 % reduction in Epac1 membrane localisation. Furthermore, the knockdown of β-arrestin2 resulted in significant attenuation of H-Ras activation, demonstrating that Epac1's interaction with β-arrestin2 is essential for H-Ras signalling following β_1-AR stimulation (Berthouze-Duquesnes et al. 2013).

Another role for β-arrestin in the regulation of the activity of GTPases was discovered during a study of the type III TGF-β receptor (TGF-βRIII) (Mythreye

and Blobe 2009). In this case, β-arrestin2 was found to mediate endocytosis and signal termination of TGF-βRIII. It was established that the TGF-βRIII interaction with β-arrestin2 was required to activate the GTPase Cdc42 in order to inhibit migration of cancer cells. Expression of wild-type TGF-βRIII leads to suppression of migration, whereas expression of the mutant TGF-βRIII-T841A (which is unable to bind β-arrestin2) did not. TGF-βRIII expression led to more than a threefold increase in Cdc42 activation relative to non-stimulated ovarian cancer cell line Ovca429. Contrastingly, TGF-βRIII-T841A-expressing cells were unable to activate Cdc42. The importance of β-arrestin2 was further confirmed through siRNA knockdown, where the co-expression of TGF-βRIII with β-arrestin2 siRNA was unable to suppress migration or activate Cdc42.

All of the research described above represents ever-accumulating evidence that supports the notion that β-arrestin is a key player in the ubiquitous regulation of GTPase activity. Many of the GAPs or GEFs, which are interacting with β-arrestin to mediate the regulation of GTPases, still remain to be discovered. However, it is already apparent that the membrane translocation of β-arrestins and their scaffolding functions play a major role in the regulation of small GTPases. This branch of β-arrestin-biased signalling has already shown to be fundamental in a variety of human diseases; therefore, targeting this signalling pathway is likely to produce a number of novel therapeutic avenues that remain untapped.

3 Physiological Relevance of β-Arrestin and Small GTPase Interaction

β-Arrestin-mediated regulation of small GTPases is obviously important for the maintenance of normal physiological function in the human body. As previously mentioned, small GTPases are molecular switches, which drive a number of signalling cascades, but they were first identified due to their oncogenic properties. Indeed, mutant versions of human Ras genes are found in around 30 % of human neoplasms. Ras gene mutations are found in 90 % of pancreatic, 30 % of lung and 50 % of colorectal carcinomas (Takai et al. 2001). An effector of Ras GTPases, Ral–GDS, has been shown to be inhibited by dominant-negative Ras, highlighting that Ral is a downstream component of Ras signalling (Kikuchi et al. 1994). However, Ral–GDS interaction with β-arrestin1 was shown to activate RalA in a manner that was independent of Ras. This process is essential for the chemotaxis of fMLP-stimulated human polymorphonuclear neutrophilic leukocytes (PMNs). The coordinated migration and granule release by PMNs is a fundamental physiological process of the innate immune system for fighting infectious bacteria (Bhattacharya et al. 2002). The physiological importance of β-arrestin/Ral–GDS/Ral signalling pathway was further highlighted following study of LPA-induced migration and invasion of human breast cancer cells. Moreover, expression levels of β-arrestin2 and Ral–GDS were increased in these transformed cells (Li et al. 2009a, b). Given

the similarities and requirements of major cytoskeletal reorganisation in chemotactic immune responses and cancer cell invasion, the role of this signalling cascade may be prevalent in the aetiology of several other diseases.

The role of β-arrestin's regulation of the Rho family GTPases links this signalling pathway with a number of physiological processes. For example, β-arrestin-mediated activation of RhoA was shown to be essential for inducing stress fibre formation following Ang II stimulation, a process known to occur in hypertrophic cardiomyocytes (Barnes et al. 2005). The importance of β-arrestin regulation of hypertrophic responses has recently been further demonstrated through its ability to differentially bind Epac1 or PDE4D5 following β-AR stimulation in order to elicit either non- or pro-hypertrophic responses (Berthouze-Duquesnes et al. 2013). β_2-AR stimulation was also shown to modulate the early phase β-arrestin-dependent activation of MAPK signalling pathways via Rac1, which may play a key role in normal cardiophysiology (Gong et al. 2008). In addition, β-arrestin is known to play an inhibitory role in cancer progression via TGF-βRIII signalling and subsequent activation of Cdc42, a GTPase shown to both suppress and promote cell migration (Mythreye and Blobe 2009). The regulation of Rac1 activity by β-arrestin was shown to be essential in regulating endothelial barrier function elicited by the anticoagulant APC (Soh and Trejo 2011). Taken together, the ubiquitous expression of β-arrestins and their regulation of small GTPase activity make them an ideal target for developing new therapeutic strategies for the treatment of diseases such as cancer, cardiac and inflammatory disorders.

4 Therapeutic Targeting of β-Arrestin and Small GTPase Interaction

As the link with GTPases is a relatively new branch of β-arrestin research and the mechanisms of GTPase are still being investigated, there has been no therapeutic targeting of this signalling pathway. A number of studies have alluded to the knockdown of β-arrestin as a therapeutic strategy; however, given the ubiquitous expression of β-arrestins in many physiological systems and the fact that cell-type-specific gene therapy is still in its infancy, then this mechanism for modulating negative outcomes following chronic β-arrestin activation of small GTPase signalling is some way off. One possible means of interfering with the β-arrestin/GTPase signalling axis is via highly specific disruption of protein–protein interactions. This was initially attempted by Li et al. (2009a, b), where they stably expressed a sequence containing the minimum binding site of Ral–GDS required to bind β-arrestin (Ral–$GDS^{616–768}$) in breast cancer cells. The overexpression of this short peptide, which competes for binding with endogenous Ral–GDS for the docking site on β-arrestin, results in inhibition of RalA activation and inhibition of cell invasion into a three-dimensional matrix. The ability of this short peptide to inhibit migration was comparable to the expression of dominant-negative RalA and

represents a bona fide molecular target for treating cancer cell invasion. This strategy is especially apt for breast cancer, as the disruption of this pathway also resulted in such cells adopting a morphology that was typical of non-malignant cells.

The β-arrestin/Ral–GDS disruptor mentioned previously, relied on ectopic expression of a competing peptide, an approach that would lend itself to gene therapy techniques for future development. However, a similar outcome can be achieved using short, cell-permeable peptides that can be simply administered to cells (Anthony et al. 2011). This approach was used to disrupt the inhibitory action of β-arrestin on ARHGAP21, which, in turn, resulted in the inhibition of Ang II-mediated RhoA activation and a reduction in stress fibre formation (Anthony et al. 2011). The cell-permeable peptide used in this case was a 25mer spanning the β-arrestin1-binding domain of the ARHGAP21 GAP domain. These disruptor peptides contained a stearic acid group attached to the N-terminal end to enable transmembrane passage of the peptide. Successful cell penetration by the peptides resulted in significantly reduced stress fibre formation and an attenuated hypertrophic response.

These types of disruptor peptides that target protein–protein interactions work well in cell-based assays (Sin et al. 2011), and this has been particularly useful for probing the functionality of β-arrestin's interaction with a host of partner proteins including MEK1 (Meng et al. 2009), JNK (Li et al. 2009a, b), PDE4D5 (Bolger et al. 2006) and Epac1 (Berthouze-Duquesnes et al. 2013). However, there are key limitations to using peptides in an in vivo system. These limitations include poor bioavailability and biodistribution, low stability in plasma due to sensitivity to proteolytic enzymes and poor ability to cross physiological barriers (Vlieghe et al. 2010). Nonetheless, there has been a revival in interest in peptide-based drugs in recent years due to the availability of chemical synthesis platforms for peptide production. There are strategies to improve the biological activity of peptides such as swapping endogenous residues of non-natural amino acids, an example being D-isomer substitutions, which protects peptides against proteolytic degradation. The blocking of N- or C-terminal ends by N-acylation, C-amidation, etc., or through the addition of carbohydrates, i.e. glycosylation, can protect against exopeptidases. N-terminal esterification or pegylation can also enhance stability and reduce immunogenicity, and pegylation can also reduce renal clearance of larger peptides. There are also many advantages to peptide-based drug candidates, as they offer great efficacy, selectivity and specificity over small molecules. The degradation products are amino acids; therefore, there is much lower risk of toxicity, and peptide-based drugs have a short half-life so they rarely accumulate in tissues (Vlieghe et al. 2010). It is possible that all β-arrestin1/GTPase pathways described above could be targeted by agents that affect protein–protein interactions, and we await new exciting developments in this area.

Having looked at the various mechanisms by which β-arrestin can modulate small GTPase activity, it is probable that a number of interacting partners remain to be found. Indeed, several are suggested in a wide-ranging proteomics study of the binding partners for β-arrestin1 and β-arrestin2 (Xiao et al. 2007). If verified, these

could represent novel targets for peptide-based or small-molecule disruptors, which may have therapeutic potential for the treatment of human disease.

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GPCRs and Arrestins in Airways: Implications for Asthma

Raymond B. Penn, Richard A. Bond, and Julia K.L. Walker

Contents

Abstract The obstructive lung disease asthma is treated by drugs that target, either directly or indirectly, G protein-coupled receptors (GPCRs). GPCRs coupled to Gq are the primary mediators of airway smooth muscle (ASM) contraction and

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increased airway resistance, whereas the Gs-coupled beta-2-adrenoceptor (β_2AR) promotes pro-relaxant signaling in and relaxation of ASM resulting in greater airway patency and reversal of life-threatening bronchoconstriction. In addition, GPCR-mediated functions in other cell types, including airway epithelium and hematopoietic cells, are involved in the control of lung inflammation that causes most asthma. The capacity of arrestins to regulate GPCR signaling, via either control of GPCR desensitization/resensitization or G protein-independent signaling, renders arrestins an intriguing therapeutic target for asthma and other obstructive lung diseases. This review will focus on the potential role of arrestins in those GPCR-mediated airway cell functions that are dysregulated in asthma.

Keywords Airway • Arrestin asthma • Beta-2-adrenoceptor • Beta-agonist • Beta-blocker • Biased agonism • Desensitization • Epithelium • G protein-coupled receptor • G protein-coupled receptor kinase • Inflammation • Protease-activated receptor • Tachyphylaxis • T cell

The lung is a highly complex organ system comprised of multiple cell types. Its primary function is to provide a conducting pathway (airways) and an interface (alveoli) to enable gas exchange: delivery of oxygen from ambient air to blood in exchange for carbon dioxide. Airways in the lung are the conduits of airflow. In the disease of asthma, airway conductance can be reduced (i.e., airway resistance is increased) by pathogenic mechanisms that cause constriction or blockage of the airway.

Lung inflammation caused by an exaggerated immune response to allergen is the most common cause of asthma. Numerous cells participate in allergic lung inflammation, including airway epithelial cells and invading inflammatory blood cells. Airway smooth muscle (ASM), which surrounds conducting airways and whose contractile state influences airway diameter and thus airway conductance, is both a target and mediator of allergic lung inflammation.

Almost every cell in the lung has an important function that is regulated by G protein-coupled receptors (GPCRs). Thus, arrestins, through their capacity to either regulate GPCR desensitization/resensitization (Chapters "Arrestin Interactions with G Protein-Coupled Receptors," "β-Arrestins and G Protein-Coupled Receptor Trafficking") or mediate G protein-independent signaling (Chapters "Quantifying Biased β-Arrestin Signaling," "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications," "Arrestin-Dependent Activation of ERK and Src Family Kinases," "Arrestin-Dependent Activation of JNK Family Kinases," "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences," "Arrestin-Dependent Localization of Phosphodiesterases," "Arrestins in Apoptosis"), are likely to play an important role in influencing airway function. This review will focus on the potential role of arrestins in those GPCR-mediated airway cell functions that are dysregulated in asthma.

1 GPCRs Play a Prominent Role in Regulating Airway Resistance in Normal Physiology and in Disease

1.1 Physiologic Control of Airway Resistance

In health and under homeostatic conditions, ASM contractile state is an important determinant of airway resistance, and GPCRs tend to control this contractile state or "tone." In both health and disease, members of the GPCR superfamily that activate the heterotrimeric G protein G_q are primarily responsible for the signaling events that promote ASM contraction, whereas GPCRs that activate G_s promote relaxation. Although ASM receives sympathetic and "nonadrenergic, noncholinergic" (van der Velden and Hulsmann 1999; Widdicombe 1998) neural input capable of modulating contractile state, release of acetylcholine from parasympathetic nerves, acting on G_q-coupled m3 muscarinic acetylcholine receptors (m3 mAChRs) on ASM, is the dominant physiologic regulator of the ASM tone (Canning and Fischer 2001; Stephens 2001) under normal conditions. Signaling events mediated by the m3 mAChR (and other G_q-coupled receptors) have been extensively characterized and include activation of phospholipase C and production of inositol 1,4,5-trisphosphate (IP3), which binds to IP3 receptors on specialized intracellular stores to promote Ca^{2+} release from these stores. This flux combines with Ca^{2+} release from ryanodine-sensitive stores and influx through plasma membrane Ca^{2+} channels to elevate $[Ca^{2+}]_i$ to levels that stimulate calcium-calmodulin, myosin light chain kinase, and ultimately myosin ATPase, leading to cross bridge cycling and sarcomere shortening [reviewed in An et al. (2007), Deshpande and Penn (2006)]. Under physiologic conditions, ASM tone is regulated primarily by the integration of afferent and efferent neural signals that determine parasympathetic release of acetylcholine (ACh); in contrast, direct effects of (counterbalancing) endogenous G_s-coupled receptor agonists appear to play little if any role. Circulating catecholamines acting on ASM β_2ARs have the potential to relax ASM, via multiple mechanisms, including antagonism of pro-contractile signaling (Billington and Penn 2003). However, in health such β_2AR activity appears of little consequence, as administration of β_2AR antagonists does not affect airway resistance in non-asthmatic subjects (Kiyingi et al. 1985; Vatrella et al. 2001). Thus, under baseline physiologic conditions, ASM tone is determined primarily by pro-contractile signaling and regulation of its stimuli, with little influence of competitive pro-relaxant stimuli. It is with the asthmatic condition where the signaling capacity and responsiveness of the β_2AR to both endogenous and exogenous beta-agonists are of importance in countering increased pro-contractile signaling and ASM tone.

1.2 Dysregulated Airway Resistance: Pathologic ASM Tone

Asthma reflects increased ASM tone. One cause of this increased tone is an exaggerated presentation of G_q-coupled receptor agonists causing increased pro-contractile signaling. Airway inflammation associated with both early and late phase allergic reactions promotes increased levels of pro-contractile agonists, acting primarily on ASM G_q-coupled receptors, that promote most asthmatic attacks. Many of these agonists (including histamine, leukotrienes, serotonin (5-HT), and some prostanoids) are secreted from numerous cell types in the airway including both infiltrating blood cells and resident airway cells. In addition, increased parasympathetic release of ACh has been shown to occur as a consequence of a loss of negative feedback inhibition by ACh acting on pre-junctional m2 mAChRs (Jacoby et al. 1993). Numerous agonists capable of activating G_i-coupled receptors are also elevated in the airway during inflammation. Although some of these agonists have direct effects on ASM, many have an indirect effect on ASM by functioning as chemokines for various cell types in the orchestration of the time-dependent inflammatory response (see below).

Unlike in the healthy lung, β_2AR activity stimulated by endogenous catecholamines does appear to be important in the asthmatic subject, as administration of βAR antagonists is not well tolerated in many asthmatic subjects (Paterson et al. 1984). Thus, under conditions of excess Gq-coupled receptor activation and increased pro-contractile signaling, ASM contractile state acquires a greater sensitivity to β_2AR activity.

Although airway inflammation is also associated with an increase in certain G_s-coupled receptor agonists in the airway, this upregulation does not appear to effectively counterbalance the increase in pro-contractile stimuli. In certain asthmatics, elevation of the COX product PGE_2 can mitigate augmented ASM pro-contractile signaling [cyclooxygenase inhibition tends to precipitate asthma attacks in sensitive patients (Morwood et al. 2005)]; however, the induction of G_q-coupled receptor agonists in the airway appears to dominate.

1.3 Most Therapeutic Approaches to Asthma Are Effective in Their Ability to Restore a Competitive Balance of Pro- and Anti-contractile Signaling

Glucocorticoids, for example, reduce the induction of pro-contractile agonists in the airway by inhibiting the influx/survival of inflammatory cells and by inhibiting the secretory actions of both inflammatory and mesenchymal airway cells. Muscarinic ACh and cysteinyl leukotriene receptor antagonists block specific G_q-coupled receptor signaling.

However, for over a century, the widely accepted first-line treatment for asthma has been β-adrenoceptor agonists (actually, the use of the indirect acting sympathomimetic, ephedrine, dates back to over 5,000 years ago but produced mixed results, and, in general, the discovery of adrenaline is recognized as the first widely accepted treatment for an asthma exacerbation). Inhaled beta-agonist activates ASM β_2ARs to stimulate PKA activity which antagonizes G_q-coupled receptor signaling at multiple junctures [reviewed in Billington and Penn (2003), Kotlikoff and Kamm (1996)]. Under most conditions, inhaled beta-agonist is effective in preventing or reversing bronchoconstriction otherwise induced by exaggerated presentation of pro-contractile agonists in the airway, demonstrating the strong regulatory capacity of the signaling events mediated by β_2ARs. Interestingly, other G_s-coupled receptors in ASM demonstrate as good if not better regulatory control of ASM contraction relative to the β_2AR. The PGE_2-activated EP2 (and possibly EP4) receptor is expressed in ASM; multiple ASM cell and tissue studies demonstrate that PGE_2 is actually more efficacious than beta-agonist in stimulating intracellular cAMP accumulation and PKA activation and in relaxing ASM tissue ex vivo. In vivo, the ability of PGE_2 to prevent/reverse bronchoconstriction appears compromised by the existence of other EP receptor subtypes (some inducing bronchoconstriction) in the lung, although a strong bronchorelaxant effect of EP2 receptor activation can be discerned from studies of EP1-4 receptor knockout mice (Tilley et al. 2003). Thus, EP2 or EP4 receptors represent a useful therapeutic target pending the development of selective receptor-subtype ligands. Presently, however, beta-agonists remain the gold standard of bronchodilators. In addition, beta-agonists may also decrease pro-contractile agents by mechanisms such as inhibiting the release of histamine from mast cells and inhibiting Ach release by activation of pre-junctional inhibitory β_2ARs from cholinergic nerves. Despite all of these advantages, βAR-agonists also have adverse events now termed the "β-paradox," and recent developments in this story will be more fully described below.

1.4 Importance of GPCR-Mediated Functions in Cell Types Other than ASM in Asthma Pathogenesis

Although the dominance of ASM in determining airway resistance renders it an obvious target of asthma therapies, the potential for developing effective asthma therapies that target GPCR-mediated functions in other cell types appears high. As will be discussed below, GPCRs that mediate allergic inflammation via the capacity to either induce inflammatory agents (in resident or invading cells) or promote inflammatory cell chemotaxis represent prime candidates for targeting.

2 Regulation of GPCR Responsiveness in Airway Function, Roles of GRKs and Arrestins in Effecting GPCR Desensitization, and the Functional Consequences

Whether regulation of GPCR responsiveness in ASM affects disease state or the response to therapy has been speculated for over 30 years. Appreciating the role of βAR agonism in regulating airway resistance, Szentinavyi (1968) originally hypothesized that asthma constituted an intrinsic defect in β_2AR function. It is now appreciated that exaggerated presentation of pro-contractile agents (discussed above) and sensitization to the calcium signal induced by these agents, and not a failure of the β_2AR to respond to endogenous catecholamines, is what drives most asthma. However, the discovery of variation in β_2AR gene (ADRB2) coding sequence prompted many to speculate that differing susceptibility to receptor desensitization could influence asthma prevalence or possibly the response to inhaled beta-agonist therapy. With respect to the former, studies to date paint a mixed picture; although some data appear to suggest ADBR2 variation may influence asthma disease progression/severity, the collective evidence indicates the more common ADRB2 polymorphisms are not clear risk factors for asthma per se. In an analysis of 8,018 asthmatics, Hall et al. (2006) concluded that although ADRB2 polymorphisms might predict a small component of the long-term prognosis in childhood asthma, they are not important determinants of asthma incidence or prevalence in the British population. A more recent consortium-based genome-wide association study of 10,265 asthmatics and 16,111 nonasthmatics did not find a genome-wide significant signal for the ADRB2 locus (Moffatt et al. 2010). With respect to response to therapy, the clinical relevance of any influence of ADBR2 variation appears minimal. Several studies [reviewed in Sayers and Hall (2005)] suggest asthmatics homozygous for the Arg16 variation have lower forced expired volumes (FEV1) and peak flow rates in response to regular inhaled albuterol, a short-acting beta-agonist. However, patients treated with long-acting beta-agonists, the more commonly prescribed maintenance asthma therapy, exhibited no genotype-specific effects on efficacy (Bleecker et al. 2007).

Although ADBR2 genotype may have minimal effect on β_2AR responsiveness in asthma, there is considerable evidence, from both clinical and basic science studies, that β_2AR desensitization is of consequence in asthma. Tachyphylaxis, defined as a loss of functional response at a more integrative level, is observed for the β_2AR in asthma as loss of the bronchoprotective effect [reviewed in Billington and Penn (2003), Deshpande and Penn (2006), Walker et al. (2011)] in asthmatics, which can be recapitulated in murine models of asthma through chronic oropharyngeal insufflation of albuterol (Lin et al. 2012). Loss of bronchoprotection has long been cited as evidence of desensitization of the β_2AR in the airway, presumably ASM β_2ARs. Moreover, chronic use of inhaled beta-agonists by asthmatics is associated with a loss of disease control (reviewed in Salpeter et al. (2006), Sears (2002), and Taylor (2009)), and even mortality (Nelson et al. (2006), Pearce et al. (1995), Spitzer et al. (1992), Stolley and Schinnar 1978). These events have resulted in a black box warning for products containing long-acting beta-agonists (LABAs).

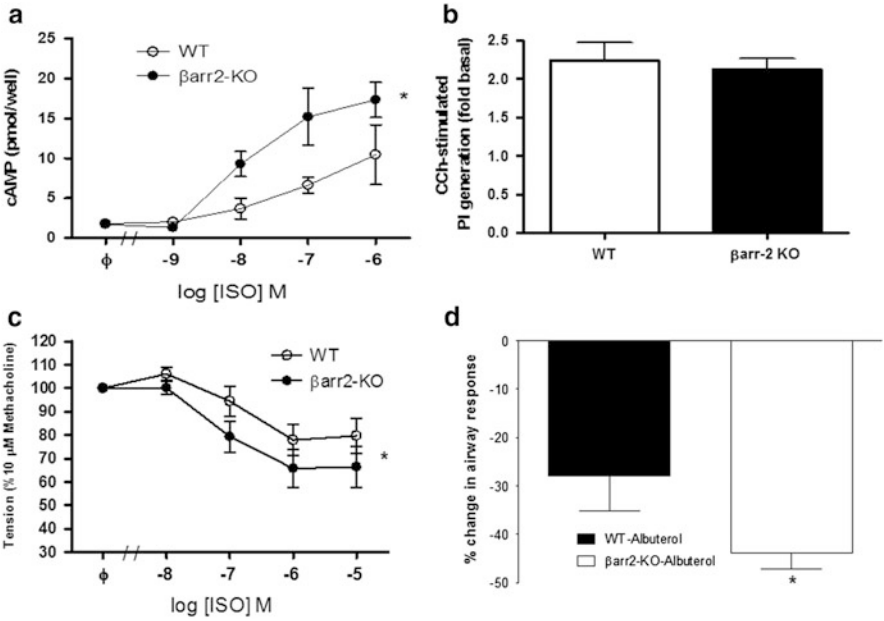

Fig. 1 β-arrestin2 specifically regulates the β_2AR signaling and function in ASM. β_2AR (**a**) but not m3 mAChR or EP2 receptor (**b** and not shown) signaling is augmented ASM cultures derived from ASM tissue from β-arrestin2 knockout mice. This increase in β_2AR is associated with an increased ability to relax carbachol-contracted ASM ex vivo (**c**) and in vivo (reflected in change in airway resistance; **d**). Data from Deshpande et al. (2008) and reproduced with permission from *FASEB Journal*

Whether these poor clinical outcomes associated with chronic beta-agonist use are a consequence of β_2AR desensitization is unclear. To more directly pursue the question of agonist-induced β_2AR desensitization in ASM and the roles of GRKs and arrestins, we undertook a series of studies. Initial studies demonstrated a clear loss of β_2AR signaling capacity, which was enhanced by GRK2 overexpression, in human ASM cultures following brief pretreatment with beta-agonist (Penn et al. 1998). Similarly, overexpression of β-arrestin1 or β-arrestin2 attenuated beta-agonist-signaling in ASM (Penn et al. 2001). We subsequently demonstrated that agonist-specific desensitization of the β_2AR, but not of the EP2 receptor, in ASM cultures could be reversed by siRNA-mediated knockdown of GRK2/3 or via expression of the (inhibitory) GRK2/3 mini-gene (Kong et al. 2008).

We subsequently employed a more comprehensive approach to explore the physiologic relevance of GRK/arrestin-dependent desensitization of ASM β_2ARs. In Deshpande et al. (2008) we demonstrated the capacity of siRNA-mediated knockdown of β-arrestin1/2 (in human ASM cultures) and/or genetic deletion of β-arrestin2 (in ASM cultures derived from β-arrestin2 knockout mice) to specifically augment β_2AR signaling (Fig. 1a, b). In analyses of ASM contraction, the ability of beta-agonist to relax contracted ASM ex vivo was greater in airways

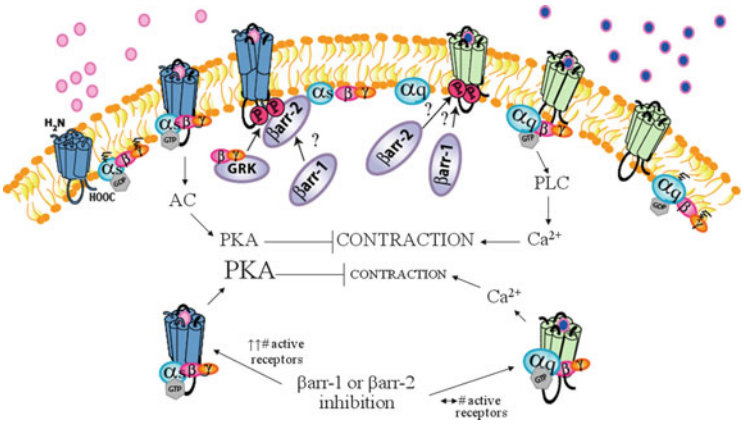

Fig. 2 Arrestin control of ASM signaling that regulates ASM contraction. β-arrestin2, and possibly β-arrestin1, limits β_2AR signaling and the capacity of beta-agonists to antagonize Gq-mediated ASM contraction. Conversely, Gq-coupled receptor (e.g., m3 mAChR or CysLT1R) signaling is minimally/not affected by arrestins. Thus, global or selective arrestin targeting has the potential to preferentially increase β_2AR signaling throughput and shift the balance of signaling resulting in reduced contractile tone

excised from β-arrestin2 knockout mice compared to airways from wild-type mice (Fig. 1c). Finally, beta-agonist (but not PGE_2) was more effective in reducing the increase in airway resistance in vivo caused by methacholine challenge (Fig. 1d). Interestingly, we observed no evidence of desensitization of mAChRs; arrestin knockdown or knockout affected neither methacholine-stimulated signaling nor methacholine-stimulated contraction of ASM ex vivo or in vivo. Although previous studies have demonstrated GRK- and arrestin-mediated desensitization of endogenous m3 mAChRs (in HEK293 cells) (Luo et al. 2008), in ASM no evidence of such at a signaling or functional level was evident. Whether this reflects a lack of m3 mAChR modification, or simply a large receptor reserve in ASM, is unknown.

An important observation that emerges from the above studies is the greater capacity of the β_2AR, relative to other GPCRs, to be subject to GRK-/arrestin-dependent desensitization in ASM. The EP2 receptor, which in ASM is more efficacious than the β_2AR in signaling and functional antagonism of contraction, exhibits minimal agonist-specific desensitization (Deshpande et al. 2008; Kong et al. 2008; Yan et al. 2011) that is unaffected by either overexpression or inhibition of GRKs/arrestins (Deshpande et al. 2008; Kong et al. 2008; Penn et al. 2001). Similarly, neither the m3 mAChR nor the cysteinyl leukotriene type 1 receptor (Deshpande et al. 2007; Naik et al. 2005) (arguably the two most clinically relevant mediators of bronchoconstriction) is subject to GRK-/arrestin-dependent desensitization in ASM. Clinically, the relative selectivity of GRKs/arrestins for the β_2AR suggests that therapies targeting GRKs or arrestins in ASM may be useful, given they would preferentially augment β_2AR (bronchorelaxant) while failing to enhance Gq-coupled receptor (pro-contractile) function (Fig. 2).

In summary, studies suggest that the β_2AR is subject to significant agonist-specific desensitization in ASM and that inhibition of arrestins can improve β_2AR signaling and the bronchodilation effect of beta-agonist. Thus, given their ability to constrain the physiologic actions of endogenous and exogenous activators of the β_2AR on ASM, arrestins likely have a role in influencing the effectiveness of asthma therapy and possibly a role in asthma occurrence/severity, via their ability to mediate β_2AR desensitization.

3 The Critical Role for Arrestins in Allergic Lung Inflammation

As noted above, numerous cell types, other than ASM, participate in the development of allergic inflammation in the lung, which is the pathologic event that causes most asthma. Therefore, via their role in regulating various GPCR-dependent functions in these cell types, arrestins have the potential to influence allergic lung inflammation and, thus, the asthma phenotype.

In 2003 Walker et al. provided insight into the role of arrestins in mediating allergic lung inflammation using a murine model in which ovalbumin sensitization and challenge (OVA S/C) was performed (Walker et al. 2003). Despite clear evidence of OVA sensitization, the asthma phenotype, as assessed by airway inflammation and hyperresponsiveness (Fig. 3a, b) and BAL fluid cytokine levels, was strikingly absent in OVA S/C β-arrestin2 knockout mice. β-arrestin-mediated regulation of ASM contraction or general immune function was ruled out by demonstrating comparable, significant elevations in airway inflammation and hyperresponsiveness in response to LPS inhalation in both WT and β-arrestin2 knockout mice.

Numerous inflammatory processes in the lung involve GPCR-dependent functions in various cell types. Among the most obvious is chemotaxis of inflammatory cells from the vascular compartment and into the airway. Once in, these cells cooperate with other invading cells, and with resident cells, to promote the inflammatory phenotype of asthma and in turn promote AHR. Given the early and important role for T cells in the asthma inflammatory cascade, further investigation revealed that T cell migration to the airways was significantly reduced in OVA S/C β-arrestin2 knockout mice (Fig. 3d) and this finding was supported by in vitro data showing that T cells devoid of β-arrestin2 exhibit a significant reduction in CCL22-mediated T cell chemotaxis (Fig. 3c). Additionally, in vitro work using naïve CD4+ T cells has shown that β-arrestin2 regulates neither polarization to nor proliferation of Th1 or Th2 cells (data not shown). Interestingly, chemotaxis of CD4+ T cells devoid of β-arrestin2 is only impaired, not abrogated; thus, it will be interesting to determine if aspects of Th2 cell function, once arriving in the airway, is also regulated by β-arrestin2.

In a follow-up study designed to explore if cell types other than CD4+ T cells are required for β-arrestin2-dependent allergic inflammation, Hollingsworth

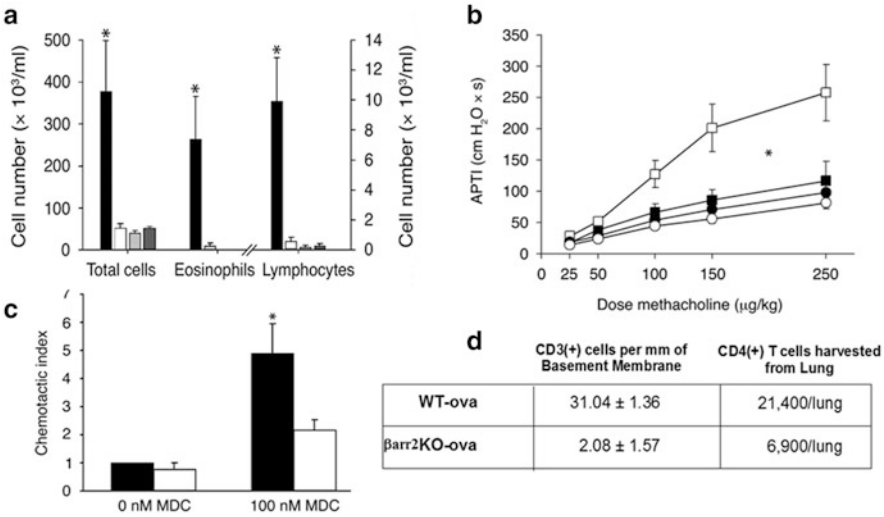

Fig. 3 β-arrestin2 is required for development of the Asthma Phenotype. OVA-treated wild-type mice (*black bars*) displayed a significant increase in (**a**) total BAL lung cells, eosinophils, and lymphocytes as well as (**b**) airway hyperresponsiveness relative to saline-treated WT and β-arrestin2-KO mice (*light* and *dark gray bars*, respectively) and compared to OVA-treated β-arrestin2-KO mice (*white bars*). (**c**) Chemotaxis of β-arrestin2-KO T lymphocytes to macrophage-derived chemokine (MDC) was significantly impaired as was (**d**) the accumulation of lung CD3+ and CD4+ T cells. Data from Walker et al. (2003) and reproduced with permission of *J Clin Invest*

et al. (2010) generated bone marrow chimera mice. OVA S/C treatment of β-arrestin2 bone marrow chimeric mice showed that β-arrestin2 expression in either hematopoietic or non-hematopoietic cells was insufficient to fully rescue the asthma phenotype. In response to IL-13 airway challenge, which can induce the signs of allergic inflammatory airway disease independent of T cells, lung eosinophilia was significantly impaired in chimeric mice that lacked β-arrestin2 expression in hematopoietic cells. Although it was not measured directly, these data suggest that in addition to T cell chemotaxis, β-arrestin2 may also regulate eosinophil chemotaxis in asthma.

4 Further Evidence Arrestins Promote Allergic Inflammation in the Lung: Honing in on the Receptors and the Potential Role of G Protein-Independent Signaling

Further mechanistic insight into how arrestins mediate allergic lung inflammation is provided by a recent study by Nichols et al (2012), which identifies the importance of protease-activated receptor 2 (PAR2) in mediating allergic lung inflammation

and distinguishes protective and pathogenic roles, respectively, for PAR2 G protein- and arrestin-dependent signaling. To assess the role for arrestin-dependent PAR2 signaling in mediating the asthma phenotype, repeated insufflation of a PAR2 agonist was combined with the OVA S/C model. PAR2 agonist administration exacerbated airway inflammation and mucin production in WT mice, but not in β-arrestin2 knockout mice, suggesting that the proinflammatory effect of PAR2 activation requires β-arrestin2. In contrast, PAR2-mediated ASM relaxation (both in vivo and ex vivo), was similar in WT and β-arrestin2 knockout mice, as were BAL levels of PGE_2, suggesting that the G protein-dependent signaling pathway mediates the anti-contractile effect of PAR2 activation through release of PGE_2.

These findings exemplify the concept of "qualitative signaling," "functional selectivity," or "biased agonism" that has emerged over the last 15 years since the discovery that arrestins could function as scaffolds and initiate signaling events distinct and independent of those promoted by heterotrimeric G proteins [reviewed in Luttrell and Gesty-Palmer (2010)]. Indeed, the mounting evidence of arrestin-mediated G protein-independent signaling by numerous GPCRs, the importance of such signaling in normal physiologic processes and disease, and the potential to selectively manipulate receptor signaling pathways via biased ligand approaches (see chapter "Quantifying Biased β-Arrestin Signaling") have ushered in a new era of GPCR biology and likely a new generation of clinical pharmacology. While the studies of the DeFea lab suggest the development of PAR2 biased ligands has the potential to differentially regulate pro- and antiasthmatic processes, recent studies by the Bond lab raise similar possibilities for a biased ligand approach for β_2ARs in the treatment of asthma.

Because β_2AR agonists are the mainstay of asthma therapy and involved in all steps of asthma management, their history and potential mechanisms merit greater detail. In 1900 adrenaline was discovered, and by 1907 it had become the first widely accepted treatment for asthma (Barnes 2006). Eventually, following seminal studies by Ahlquist in 1948 (AHLQUIST 1948a, b), subdividing adrenergic receptors into alpha- and beta-subtypes, and then Lands in 1967 subdividing beta-receptors into β_1ARs and β_2ARs, adrenaline was identified as the endogenous ligand for the β_2AR (Lands et al. 1967). In the 1950s inhaled isoproterenol was introduced as a highly effective bronchodilator with less potential for cardiovascular events like hypertension than adrenaline. Shortly after the debut of isoproterenol began the highly controversial suggestion that the β_2AR also mediated deleterious adverse effects in asthma therapy. The first β_2AR agonist "epidemic" reported was an increase in mortality associated with the use of high-dose isoproterenol in England and Wales (Stolley 1972; Stolley and Schinnar 1978). The scientific cause for this increase mortality was attributed to its nonselective agonism of both β_1ARs and β_2ARs, combined with its high efficacy, producing excessive cardiac stimulation. The next β_2AR agonist epidemic occurred in Australia and New Zealand (Grainger et al. 1991; Pearce et al. 1995) and was associated with the use of the β_2AR agonist, fenoterol, and despite fueling the "β-agonist paradox" controversy because of the failure to establish a causal effect of fenoterol, the observed spike in asthma mortality decreased following its withdrawal from the

market. Fenoterol was another fairly high-efficacy drug and perhaps continued to support the desensitization hypothesis as the cause of adverse effects. However, there were also sporadic epidemiological reports correlating the amounts of use of the rescue β_2AR agonist, albuterol (a much lower-efficacy agonist), and increased mortality. In turn these results were explained by the fact that it was much more likely that people with severe asthma required more frequent use of rescue inhalers (Sears et al. 1990).

Then in 1993 the Serevent® Surveillance Trial results showed that, while not reaching significance, a new longer-acting β_2AR agonist (LABA), salmeterol, that was administered twice daily also produced higher mortality than the placebo group which used as needed albuterol for rescue (Castle et al. 1993). These last results prompted the FDA to ask the makers of salmeterol to begin a post-marketing surveillance trial that began in 1998 (Nelson et al. 2006). This trial was a very large multicenter trial of ~13,000 patients receiving salmeterol twice daily and another 13,000 in the placebo group (which consisted of as needed rescue with albuterol). This trial was prematurely stopped due to excess mortality in the salmeterol group and began a series of FDA labeling requirements each causing further restrictions on the use of LABAs, including their removal as add-on therapy to as needed short-acting β_2AR agonist rescue. Thus, while clearly the most effective bronchodilators ever produced, and eminently useful in reversing bronchoconstriction and saving countless lives, β_2AR agonists were shadowed by reports that constant, chronic use may adversely affect asthma control and negatively impact morbidity and mortality.

It was against this background that in 2001 we formulated a hypothesis that asthma and congestive heart failure (CHF), while different in many ways, may be similar with regards to the chronic use and outcomes of β-adrenoceptor ligands (Bond 2001). In CHF, as in asthma, the use of β-agonists was associated with symptomatic improvement, and as in asthma, β-blockers were contraindicated. However, evidence was accumulating in CHF that chronic treatment with certain β-blockers resulted in decreased mortality. Finally, in 1997, the FDA approved the first β-blocker, carvedilol, for the treatment of CHF. To our knowledge, this marked the first time in history that a drug had moved from a contraindicated drug to the drug of choice for decreasing mortality in the disease it was once contraindicated.

Our studies to date show a requirement of β_2AR signaling for the development of the asthma phenotype in murine asthma models [reviewed in Walker et al. (2011)]. To date we have shown that chronic treatment with certain β-blockers attenuates AHR, inflammation, mucin overproduction, and the increase in Th2 cytokines (Callaerts-Vegh et al. 2004). We have demonstrated that the effect is mediated via the β_2AR, as treatment with the highly selective β_2AR antagonist, ICI-118,551 (Nguyen et al. 2008), or ablation of ADBR2 (Nguyen et al. 2009) prevents the development of all the measured parameters of the asthma phenotype. Furthermore, two small, proof-of-concept clinical trials have shown that chronic treatment with the β-blocker nadolol attenuates AHR (as measured by the PC20 methacholine) in mild asthmatics (Hanania et al. 2008, 2010). These results have now led to an

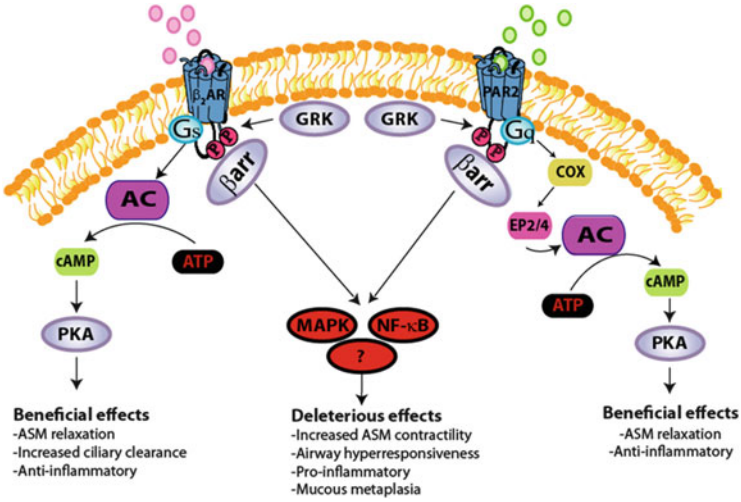

Fig. 4 Pro- and antiasthmatic effects linked to specific G protein-independent and G protein-dependent signaling, respectively. Proposed model by which qualitative signaling influences asthma pathogenesis. Based on studies by the Walker, DeFea, and Bond labs, G protein-dependent signaling mediated by either PAR2 or β_2AR receptors in various airway cell types (including ASM, airway epithelium, or invading hematopoietic cells) can result in inhibition of multiple indices of inflammation or direct relaxation of ASM, thus attenuating the asthma phenotype. Conversely, G protein-independent signaling mediated by arrestins promotes inflammation and associated airway hyperresponsiveness

ongoing NIH-sponsored double-blind, placebo-controlled, multicenter trial using nadolol in mild asthmatics (NIMA trial; ClinicalTrials.gov).

Several critical questions arise from these findings; perhaps most importantly, what is the signaling pathway(s), and in what cell type(s) are they necessary to allow the proinflammatory allergic response? Whereas the proinflammatory actions of the PAR2 receptor appear dependent on G protein-independent arrestin signaling (and PAR2-dependent (indirect) activation of Gs appears protective), the β_2AR-mediated signaling events that promote inflammation and the asthma phenotype are unclear. In ASM, Gs/PKA signaling is implicated in bronchodilation and in the inhibition of ASM growth, caused by either beta-agonists or by EP2 receptor activation. As noted, EP2 receptors do not recruit arrestins and appear resistant to GRK/arrestin-mediated desensitization (Deshpande et al. 2008; Kong et al. 2008). Only a handful of inflammatory cytokines (e.g., IL-6) are known to be positively regulated by Gs/PKA signaling [reviewed in Billington et al. (2013)]. With respect to β_2AR-mediated augmentation of mucin production in airway epithelia, Gray et al. (2004) reported that IL-1β and PGE_2-stimulated Muc5a secretion in human airway epithelial cells was blocked by cyclooxygenase inhibitors and PKA inhibitors, respectively. However, an early study suggested that cyclic AMP production stimulated by beta-agonist is dissociated from mucin secretion in rat submandibular acinar cells (Bradbury et al. 1990).

Alternatively, arrestin-dependent signaling is capable of activating several pathways (e.g., p44/p44, Akt, NF-kB, NFAT) (see chapters "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional and Therapeutic Implications," "Arrestin-Dependent Activation of ERK and Src Family Kinases," "Arrestin-Dependent Activation of JNK Family Kinases," "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences") important in inducing inflammatory gene expression in multiple cell types. Similar to that observed for the PAR2 receptor, β_2AR-mediated arrestin signaling may also play a role in promoting the inflammatory cell migration mechanism dependent on arrestin. An intriguing hypothesis suggested by the collective findings to date is that arrestin-dependent signaling, mediated by various GPCRs in multiple cells, is an important effector of multiple features of asthma, whereas G protein signaling, specifically that which results in PKA activation, has a counterbalancing effect (Fig. 4).

To date however, limited evidence exists specifically implicating arrestin signaling in asthma pathogenesis associated with β_2AR agonism. The subset of β-blockers that posses β_2AR inverse agonist properties, such as ICI-118,551 and nadolol, has been shown to be effective in attenuation of the development of allergic inflammation in murine models. However, this same subset is effective in antagonizing both Gs and arrestin-dependent signaling by the β_2AR, and thus no insight into which specific β_2AR signaling pathway promotes AHR is afforded by these studies. This dual inhibitory property of the β-blockers for both pathways also prevents us from determining if one of the pathways may be protective. Future studies employing genetic approaches in murine models, as well as testing different β_2AR ligands of varying "bias," should clarify the role of β_2AR-mediated arrestin signaling in asthma pathogenesis. Should a contributory role be discerned, either global targeting of arrestins in the lung or more selective targeting of β_2AR-mediated arrestin signaling with biased ligands that fail to promote arrestin signaling would represent innovative asthma drugs.

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Arrestins as Regulatory Hubs in Cancer Signalling Pathways

Hervé Enslen, Evelyne Lima-Fernandes, and Mark G.H. Scott

Contents

Abstract Non-visual arrestins were initially appreciated for the roles they play in the negative regulation of G protein-coupled receptors through the processes of desensitisation and endocytosis. The arrestins are also now known as protein scaffolding platforms that act downstream of multiple types of receptors, ensuring relevant transmission of information for an appropriate cellular response. They function as regulatory hubs in several important signalling pathways that are often

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dysregulated in human cancers. Interestingly, several recent studies have documented changes in expression and localisation of arrestins that occur during cancer progression and that correlate with clinical outcome. Here, we discuss these advances and how changes in expression/localisation may affect functional outputs of arrestins in cancer biology.

Keywords Arrestins • Cancer • Src • PI3K • AKT • PTEN • Mdm2 • p53 • β-Catenin • Proliferation • Migration • Invasion • Metastasis

1 Introduction

The ubiquitously expressed non-visual arrestins, β-arrestin1 (β-arr1) and β-arrestin2 (β-arr2), also known as arrestin2 and arrestin3, were initially appreciated for the roles they play in the negative regulation of G protein-coupled receptors (GPCRs), through the processes of desensitisation and internalisation (Moore et al. 2007). In addition to these negative roles in GPCR signalling, β-arrs are now also known to act as signal transducers by serving as multifunctional scaffolds, downstream of multiple different classes of receptors or even via receptor-independent mechanisms (Gurevich and Gurevich 2006; DeWire et al. 2007; Gurevich et al. 2008; Luttrell and Gesty-Palmer 2010; DeFea 2011; Shukla et al. 2011). Recent studies using high-throughput screens (yeast 2-hybrid and mass spectrometry systems) have identified many β-arr-interaction partners that are important for signal transduction, including Src, MAPKs, small GTPases, components of the PI3K/AKT signalling cascade, transcription factors and cytoskeletal proteins (Xiao et al. 2007). Through their role as scaffolds, β-arrs dynamically regulate the activities and/or subcellular distribution of these binding partners to ensure relevant transmission of information in space and time and thus an appropriate cellular response. β-arrs therefore control a broad range of cellular functions including proliferation, cytoskeletal rearrangement and cell motility (DeFea 2007; DeWire et al. 2007) (see chapters "Arrestins in apoptosis", "Molecular Mechanisms Underlying Beta-Arrestin-Dependent Chemotaxis and Actin-Cytoskeletal Reorganization"). These processes are perturbed in cancer leading to inappropriate cell growth, migration and invasion. The in vivo implication of β-arrs in the modulation of tumourigenesis has been demonstrated in mice. For example, transgenic mice overexpressing β-arr1 promoted rapid tumour progression and increased angiogenesis (Zou et al. 2008), whereas β-arr2 knockout mice displayed enhanced tumour growth and metastasis in a model of lung cancer (Raghuwanshi et al. 2008). A number of recent studies have also documented changes in the levels and/or localisation of β-arrs that correlate with progression of different types of human cancer. This chapter discusses these advances and how changes in β-arr levels/localisation

may impact on cancer progression with regard to known signalling roles attributed to these scaffolds.

2 β-Arrestin Expression Levels in Human Cancer

2.1 β-Arrestin Levels in Human Breast Cancer

One of the first studies to investigate if β-arr expression was altered in cancer examined mRNA levels of both β-arr1 and β-arr2 by quantitative real-time PCR (qPCR) in a cDNA array of tissue samples taken from breast cancer patients (Li et al. 2009) (see also Table 1 for studies documenting changes in β-arr expression in human tumours). Compared to non-invasive stage 0 breast cancer samples, β-arr2 transcripts were significantly increased in the invasive stages I to IV. In the same samples, mRNA levels for the lysophosphatidic acid (LPA) LPA1R and LPA2R GPCRs and Ral GTPases, which are all known to play key roles in cancer progression, were also upregulated. Although β-arr1 transcripts demonstrated an increased trend in stages I to IV versus stage 0, the effect was not statistically significant.

Subsequently, two other studies investigated the levels of β-arr protein expression during breast cancer progression using large cohorts with available clinicopathological parameters (Lundgren et al. 2011; Michal et al. 2011). In the first study, Michal et al. used both immunohistochemistry (IHC) and immunofluorescence (IF) to determine the localisation of β-arrs in normal breast tissue with β-arr1- and β-arr2-specific antibodies. Mammary epithelia contain two cell types: the luminal cells that form the inner layer of mammary ducts and the contractile myoepithelial cells, which surround the polarised luminal compartment and form contacts with the stroma. Both β-arr1 and β-arr2 were expressed in luminal and myoepithelial cells. Whereas β-arr2 showed similar levels between both cell types, β-arr1 was more concentrated in myoepithelial cells. A quantitative IF (qIF) approach to assess the expression of β-arrs during cancer progression was also used in this study. β-arr1 expression was found to decrease from non-invasive ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) and again to lymph node metastasis. In contrast, β-arr2 expression increased during invasive breast cancer progression, with stronger staining obtained in IDC or metastatic lymph node samples compared to DCIS or normal tissue.

Breast carcinomas can be classified into different molecular subtypes based on data obtained from cDNA microarray and IHC analyses (Eroles et al. 2012). These include luminal A (Oestrogen receptor (ER) +, Progesterone receptor (PR) +, Human epidermal growth factor receptor 2 (HER2) −) and luminal B (ER+/−, PR+/−, HER2−/+) subtypes, triple negative (ER−, PR−, HER2−) and HER2-enriched (ER−, PR−, HER2+). Patients with HER2-enriched and triple negative subtypes have the worst prognosis. Decreased β-arr1 expression was found to

Table 1 Studies documenting changes in β-arrestin expression in primary human tumours

Cancer	Arrestin expression levels		Technique	Associated clinical characteristics	Cohort size	Implicated signalling pathway	Reference
	β-arr1	β-arr2					
Breast	Increase	(Non-significant increase)	qPCR	↑ β-arr2 transcripts in advanced cancer (stages I–IV) versus stage 0 non-invasive cancer	48	LPAR/β-arr/Ral	Li et al. (2009)
Breast	Decrease	Increase	qIF	↓ β-arr1 and ↑ β-arr2 levels in luminally derived tumours associated with ↓ survival	651	N.D.	Michal et al. (2011)
Breast	Increase	N.D.	qIF	↑ β-arr1 expression in IDC and mIDC. VEGF expression positively correlated with β-arr1	79	β-arr1/HIF-1/VEGF	Shenoy et al. (2012)
Breast	High or low	N.D.	IHC	Low or high β-arr1 expression levels in tumour stroma predicts clinical outcome	Cohort I: 179 Cohort II: 564	N.D.	Lundgren et al. (2011)
Glioblastoma	Increase total β-arr1 Decrease p-β-arr1: total β-arr1	N.D.	IHC, WB	↓ p-β-arr1 correlates with poor survival in grade IV glioblastoma	126	EGFRvIII/β-arr1/Src/ERK	Mandell et al. (2009)
ALL	Increase	N.D.	qPCR, WB	↑ β-arr1 expression positively related with risk stratification. Notch 1 expression inversely correlated with β-arr1	155	N.D.	Liu et al. (2011)
Prostate	N.D.	Inverse correlation between β-arr2 and AR levels	IHC	N.D.	24	β-arr2/Mdm2/AR destruction complex	Lakshmikanthan et al. (2009)
Ovary	Increase	N.D.	IHC	Increased co-expression of β-arr1 with ETAR in advanced cancer stages (grades 3–4)	35	FTAR/β-arr/Src/β-catenin ETAR/β-arr/Axin	Rosano et al. (2009)
Lung	Increase total β-arr1 Increase nuclear localisation	N.D.	IHC	↑ total and nuclear expression of β-arr1 in squamous cell carcinoma, adenocarcinoma and bronchio-alveolar carcinoma	50	nAchR/β-arr1/Src/AKT/E2F	Dasgupta et al. (2011)

Abbreviations used: *ALL* Acute lymphoblastic leukaemia, *AR* Androgen receptor, *EGFRvIII* EGFR variant III, *ETAR* Endothelin A receptor, *E2F* E2F transcription factor, *IHC* Immunohistochemistry, *LPAR* Lysophosphatidic acid receptor, *nAChR* nicotinic acetylcholine receptor, *N.D.* Not documented, *N.S.* Not significant, *qIF* quantitative Immunofluorescence, *qPCR* quantitative real-time PCR, *WB* Western blot

correlate with ER- and triple negative status in addition to clinical parameters of enhanced tumour aggressiveness, such as increased nuclear grade, tumour size and positive lymph node status (Michal et al. 2011). The only statistically significant association for β-arr2 was in HER2+ patients who displayed lower β-arr2 levels. Finally, decreased β-arr1 and increased β-arr2 levels were shown to be associated with poor clinical outcome. Combined, the data from this study show that β-arr1 expression decreases while β-arr2 increases during breast cancer progression and this is linked to reduced patient survival.

In the second study investigating β-arr protein expression in breast cancer, Lundgren et al. used a β-arr1-specific antibody to evaluate β-arr1 levels in two different patient cohorts (Lundgren et al. 2011). β-arr1 displayed a cytoplasmic distribution in tumour cells and was also detected in stromal cells, including fibroblasts, endothelial and various immune cells.

In cohort I, both tumour and stromal β-arr1 expression were inversely correlated with ER expression and positively correlated with HER2 status. Stromal β-arr1 expression was further positively correlated with clinical parameters of enhanced tumour aggressiveness including histological grading, tumour size, lymph node status, distant metastases and the hypoxia marker, carbonic anhydrase IX. Positive association between tumour cell and stromal cell β-arr1 expression was also found. In cohort II, both tumour and stromal β-arr1 expression were inversely correlated with ER and PR expression, and tumour β-arr1 was positively correlated with HER2 status. Tumour cell expression of β-arr1 was positively correlated with histological grade, and stromal β-arr1 expression was positively correlated with histological grade, tumour size, proliferation (Ki-67 staining) and hypoxia-inducible factor-1α (HIF1α). As with cohort I, cohort II also showed positive association between tumour cell and stromal cell β-arr1. In cohort I high levels of stromal β-arr1 were associated with decreased patient survival and in cohort II both high and negative stromal expression was correlated with poor clinical outcome compared to patients with low or moderate expression. Interestingly, in cohort II only patients with low or moderate stromal β-arr1 expression benefited from tamoxifen treatment compared to patients with high or absent expression. This study therefore demonstrates that β-arr1 tumour stromal expression can predict clinical outcome as well as response to tamoxifen in breast cancer.

Finally, a further study documented that β-arr1 protein levels increased from normal breast tissue to IDC and metastatic IDC (Shenoy et al. 2012). A positive correlation between protein expression levels of β-arr1 and the angiogenic factor vascular endothelial growth factor (VEGF) was found in IDC samples. β-arrs are known to shuttle between cytoplasmic and nuclear compartments. While both β-arr1 and 2 can be actively imported into the nucleus, β-arr2 is rapidly excluded via CRM1-dependent nuclear export due to the presence of a nuclear export signal (NES) that is absent in β-arr1 (Scott et al. 2002; Wang et al. 2003a). A nuclear localisation signal (NLS) was recently identified in β-arr1 (Hoeppner et al. 2012). In the nucleus, β-arr1 plays roles in transcriptional control (Kang et al. 2005; Ma and Pei 2007) and the NLS was shown to be important for its nuclear distribution and transcriptional roles. Using breast cancer cells as a model, Shenoy et al. found that

during hypoxic conditions β-arr1 strongly interacts with HIF1α, the master transcriptional switch during hypoxia, whereas cytoplasmic β-arr2 or β-arr1 carrying a "gain of function" NES, rendering it cytoplasmic, did not. Under hypoxic conditions nuclear β-arr1 promotes transcription of a luciferase reporter under the control of the hypoxia response element (HRE) derived from the 5′-UTR of the VEGF gene, suggesting that β-arr1 may promote survival of breast cancer cells via VEGF signalling. Interestingly, β-arr can also act downstream of activated VEGF receptor to promote the endocytosis of VE-cadherin leading to increased endothelial permeability, which is characteristic of cancer progression (Gavard and Gutkind 2006).

These studies therefore indicate that increased, decreased or absent β-arr expression correlates with breast cancer progression. These differences may seem somewhat conflicting, but they most likely reflect the critical role of β-arr concentration to perform their roles as scaffolds, bringing together the correct protein partners in space and time depending on the associated biological context (Gurevich and Gurevich 2012). Changes in β-arr levels may therefore lead to impaired β-arr signalling or skew one pathway in favour of another, leading to disease progression.

2.2 β-Arrestin Levels in Other Types of Human Cancer

Changes in β-arr levels have also been documented in studies investigating acute lymphoblastic leukaemia (ALL), gliomas and lung, prostate and ovarian cancers (Lakshmikanthan et al. 2009; Mandell et al. 2009; Rosano et al. 2009; Dasgupta et al. 2011; Liu et al. 2011). ALL is the most common form of childhood cancer. ALL patients can be classified into different risk groups: standard risk (SR), medium risk (MR) and high risk (HR) so they can be given appropriate therapy. Levels of β-arr1 mRNA were significantly increased in mononuclear cells of bone marrow and periphery blood in paediatric ALL patients versus controls (Liu et al. 2011). Furthermore, β-arr1 expression was positively correlated with white blood cell count and increased risk classification, with β-arr1 mRNA levels being more than twice as high in the HR group than in the SR group. Using Western blot and IF, the authors also demonstrated that β-arr1 protein levels were increased in ALL patients versus non-leukaemia controls. These data therefore suggest that β-arr1 may participate in the progression of paediatric ALL.

The endothelin-A receptor (ETAR) GPCR, which is regulated by β-arrs, plays a critical role in ovarian tumourigenesis and progression (Rosano et al. 2009). The ETAR and its ligand ET-1 are overexpressed in primary and metastatic human ovarian carcinomas. A cohort of ovarian tumours was examined for ETAR and β-arr1 expression by IHC. The levels of ETAR and β-arr1 and their co-expression increased with higher tumour grades (Rosano et al. 2009).

Dysregulation of the expression and/or function of the androgen receptor (AR), a nuclear hormone receptor, contributes to the initiation of prostate cancer and transition to the hormone-refractory disease. IHC studies on microarrays of prostatectomy samples demonstrated that glands that expressed β-arr2, in general, did

not express AR and vice versa, suggesting an inverse correlation between β-arr2 and AR expression (Lakshmikanthan et al. 2009). Furthermore, the authors also demonstrated that levels of prostate-specific antigen (a readout for AR activity) were also inversely correlated.

Total levels of β-arr1 and its subcellular localisation were monitored in a lung cancer tissue microarray (Dasgupta et al. 2011). Both primary and metastatic squamous cell carcinomas and bronchio-alveolar carcinomas as well as metastatic adenocarcinoma displayed increased total levels of β-arr1, when compared to matched distant normal lung tissue. Highest levels of nuclear β-arr1 were observed in the metastatic cancers. These results suggest that increased β-arr1 expression and nuclear localisation may contribute to non-small cell lung cancer (NSCLC) progression.

Serine 412 (Ser 412) within the C-terminal tail of β-arr1 is an important regulatory phosphorylation site for β-arr1 scaffolding function (Luttrell et al. 1999). Dephosphorylation of Ser 412 promotes binding of β-arr1 to c-Src (see Sect. 3.1). β-arr1 phosphorylation status and total protein levels were investigated in human glioma samples using anti-phosho-Ser 412- and total β-arr1 antibodies (Mandell et al. 2009). A strong negative correlation between glioma grade and phospho-β-arr1 was observed, with the majority of glioblastoma samples showing complete loss of phospho-β-arr1 staining in tumour cells. Decreased phospho-β-arr1 levels also correlated with decreased survival.

Taken together, the above studies indicate that, although likely implicating various pathophysiological mechanisms, changes in levels (both upregulation and downmodulation), localisation or post-translational modifications of β-arrs correlate with different types of cancer progression and thus clinical outcome.

3 β-Arrestin Scaffolds in Cancer Signalling Pathways

Changes in expression and/or localisation of β-arrs would have important consequences for processes that often go awry in cancer such as proliferation and cell migration. From a molecular point of view, alterations in β-arr levels would impact on the desensitisation and internalisation of GPCRs, such as CXCR4, LPAR and PAR2, which are involved in tumour growth, angiogenesis and metastasis (Balkwill 2004; Schaffner and Ruf 2009; Houben and Moolenaar 2011). β-arrs can also be localised to many subcellular compartments, including the cytoplasm, plasma membrane, endosomes, nucleus, centrosome, primary cilium, membrane ruffles and pseudopodia. Dynamic changes in localisation of β-arrs are of paramount importance by redirecting scaffold-associated activities, from one cellular compartment to another. A good example of the functional consequences of β-arr-mediated spatially sequestered signalling is in the regulation of cell shape and motility (see chapter "Molecular Mechanisms Underlying Beta-Arrestin-Dependent Chemotaxis and Actin-Cytoskeletal Reorganization"). β-arrs achieve this by controlling the activity of the small GTPases (see chapter "Arrestin Regulation of Small

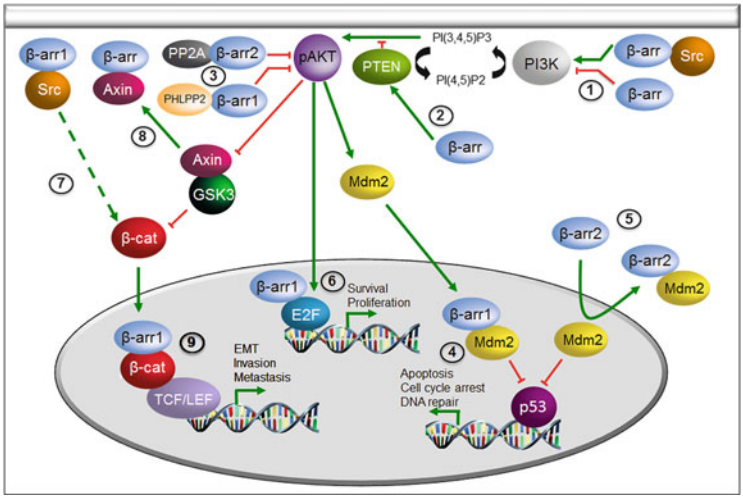

Fig. 1 β-arrestins as signalling hubs in cancer signalling pathways. (1) β-arr1 and 2 can either enhance or inhibit PI3K activation depending on the receptor that is activated. PI3K activation results in increased membrane phosphatidylinositol (3,4,5) tris-phosphate (PI(3,4,5)P3) accumulation promoting membrane recruitment and phosphorylation/activation of AKT (pAKT). Note that receptors at the cell membrane are not shown. (2) β-arr1 and 2 can both stimulate the lipid phosphatase activity of PTEN, resulting in the conversion of PI(3,4,5)P3 to PI(4,5)P2 and inhibition of PI3K/AKT signalling. (3) β-arrs can recruit protein phosphatases to AKT to limit its signalling. β-arr1 can form a complex with the phosphatase PHLPP2 and AKT, while β-arr2 can form a complex with the phosphatase PP2A. (4) Following β-arr1-mediated activation of AKT, Mdm2 is phosphorylated and translocates to the nucleus. Nuclear β-arr1 then scaffolds Mdm2 to p53, promoting its degradation and leading to impaired genomic integrity. (5) β-arr2 undergoes constitutive cytonuclear shuttling allowing it to relocalise Mdm2 from the nucleus to the cytoplasm. This results in increased p53 stability, cell cycle arrest and apoptosis. (6) β-arr1 activation of Src, and subsequent AKT activation, is associated with translocation of β-arr1 into the nucleus. β-arr1 is recruited to E2F-responsive promoters where it regulates histone acetylation and increased transcription of pro-survival and proliferative genes. (7) β-arr1 activation of Src causes transactivation of the EGFR and phosphorylation of β-catenin (β-cat) promoting a nuclear β-cat/TCF complex. (8) In a parallel signalling pathway β-arrs bind axin, leading to the release and inactivation of GSK3, and increased β-cat stabilisation. (9) β-arr1 can form a complex with β-cat in the nucleus. The β-arr1–β-cat complex increases transcription of genes implicated in cell migration, invasion and EMT

GTPases") RhoA, Cdc42 and RalA (Min and Defea 2011) in space and time, as well as scaffolding important cytoskeletal proteins such as the actin binding protein filamin (Scott et al. 2006) and the actin filament severing protein cofilin (Zoudilova et al. 2007). Several studies have shown that these properties of β-arrs regulate polarity, cytoskeletal reorganisation, migration and invasion in ovarian and breast cancer cell line systems (Li et al. 2009; Mythreye and Blobe 2009; Min and Defea 2011). β-arrs also control a number of important signal transduction pathways that are frequently targeted in cancer, including the Src/MAPK, PTEN/PI3K/AKT, Mdm2/p53 and β-catenin pathways (see Fig. 1 and Table 2) (see also chapters "Arrestin Interaction with E3 Ubiquitin Ligases and Deubiquitinases: Functional

Table 2 Partial list of β-arrestin-mediated functional outputs in cancer signalling pathways

Arrestin involved	Receptor	Signalling effectors	Experimental model	Functional output	Reference
β-arr1	EP4	Src, AKT	Colorectal carcinoma cells and nude mice	↑ Cell migration and metastasis	Buchanan et al. (2006)
β-arr2	CXCR2	NF-κB	Lewis lung cancer cells and β-arr2−/− mice	↓ Tumour growth and metastasis	Raghuwanshi et al. (2008)
β-arr1	nAChR	Src, AKT, E2F	NSCLC cells	↑ Cell proliferation and survival	Chellappan et al. (2011)
β-arr1	–	PI3K, MMP9, VEGF	Hepatoma or lymphoma cells and β-arr1 transgenic mice	↑ Tumour angiogenesis and progression	Zou et al. (2008)
β-arr1 and 2	LPA1R	Rho, ROCK, PTEN	β-arr1−/−β-arr2−/−MEFs, Prostate cancer cells, glioma cells	↓ Cell proliferation and ↑ Cell migration	Lima-Fernandes et al. (2011)
β-arr1	β2-AR	AKT, Mdm2, p53	Osteosarcoma cells and β-arr1−/− mice and MEFs	↑ DNA damage	Hara et al. (2011)
β-arr2	–	Mdm2, p53	Osteosarcoma and NSCLC cells	↑ Cell cycle arrest and apoptosis	Wang et al. (2003a, b), Boularan et al. (2007)
β-arr2	AR	Mdm2	Prostate cancer cells	↑ AR degradation	Lakshmikanthan et al. (2009)
β-arr1 and 2	ETAR	Src, AKT, β-catenin	Ovarian cancer cells and nude mice	↑ Invasions metastasis	Rosano et al. (2009, 2012)
β-arr2	Wnt pathway	β-catenin	ApcΔ14/+β-arr2−/− mice	↑ Intestinal tumourigenesis	Bonnans et al. (2012)
β-arr2	Wnt pathway	β-catenin	Haematopoietic stem cells from β-arr2−/− mice	↑ Myeloid leukaemia	Fereshteh et al. (2012)
β-arr1 and 2	LPA1R	RaIA	Breast cancer cells	↑ Migration and invasion	Li et al. (2009)
β-arr2	TβRIII	Cdc42	Ovarian and breast cancer cells	↓ Polarity and migration	Mythreye and Blobe (2009)

Abbreviations used: *AR* Androgen receptor, *β2AR* β2 adrenergic receptor, *EP4* Prostaglandin E receptor 4, *ETAR* Endothelin A receptor, *E2F* E2F transcription factor, *LPA1R* Lysophosphatidic acid receptor 1, *MEF* mouse embryonic fibroblasts, *nAChR* nicotinic acetylcholine receptor, *NSCLC* non-small cell lung cancer, *TβRIII* Type III TGF-β receptor

and Therapeutic Implications", "Arrestin-Dependent Activation of ERK and Src Family Kinases", "Arrestin-Dependent Activation of JNK Family Kinases", "Arrestin-Mediated Activation of p38 MAPK: Molecular Mechanisms and Behavioral Consequences", "Arrestins in Apoptosis"). Differences in expression may therefore also affect signal outputs of β-arr-mediated scaffolds in these pathways. Here, we will discuss the roles of β-arr scaffolds in the regulation of these pathways and their relation to cancer biology.

3.1 β-arr–Src Scaffolds

The first non-GPCR signalling partner identified for β-arr was the oncoprotein Src (Luttrell et al. 1999). β-arr was found to scaffold and activate Src downstream of the β2 adrenergic receptor. β-arr–Src scaffolds can influence downstream activation of AKT and the ERK1/2 MAPKs (see chapter "Arrestin-Dependent Activation of ERK and Src Family Kinases"). β-arrs have also been shown to scaffold multiple components of MAPK cascades downstream of numerous GPCRs to control cell proliferation (DeFea et al. 2000a, b; Luttrell et al. 2001). In prostate cancer cells, a β-arr2–Src complex was increased following β2 adrenergic receptor stimulation and this was associated with increased cell proliferation (Zhang et al. 2011). The interaction between Src and β-arr1 is regulated by phosphorylation. Phosphorylation of Ser412 on β-arr1 (absent in β-arr2) inhibits Src binding and this was shown to inhibit ERK1/2 signalling (Luttrell et al. 1999). β-arr1-mediated activation of Src was shown to be important for EGFR transactivation, downstream AKT activation and subsequent migration and metastasis of colorectal carcinoma cells (Buchanan et al. 2006). Cells overexpressing a Ser412Asp mutant of β-arr1, which mimics phosphorylated β-arr1 and that displays impaired binding to Src, exhibited both decreased cell migration in vitro and metastasis in vivo compared to cells expressing wild-type β-arr1 that metastasise at a high rate. Similarly, β-arr1–Src scaffolds were reported to be involved in lung cancer cell migration in vitro and in vivo in mouse skin papilloma development (Chun et al. 2009). Several glioblastoma cell lines displayed reduced phosphorylation of β-arr1 in agreement with findings observed in human glioma samples in the same study (Mandell et al. 2009; see Sect. 2.2). Glioblastomas frequently overexpress the EGFR variant III (EGFRvIII), which is characterised by a truncated extracellular domain and ligand-independent constitutive activity. Oncogenic signalling via EGFRvIII leads to increased cell proliferation, invasion and resistance to apoptosis. In glioma U251 cells, β-arr1 was dephosphorylated downstream of the constitutively active EGFRvIII and this was associated with increased ERK1/2 activation (Mandell et al. 2009), in agreement with results demonstrating that a Ser412Ala mutant of β-arr1 gives increased ERK signalling compared to a Ser412Asp mutant, which mimics the phosphorylated state (Luttrell et al. 1999). Finally, following nicotine-induced activation of the nicotinic acetylcholine receptor in NSCLC cells, β-arr1–Src signalling is associated with the translocation of β-arr1 into the nucleus

(Dasgupta et al. 2011), where it is recruited to promoters of E2F transcription factor-responsive proliferative and pro-survival genes to facilitate histone acetylation. In NSCLC tumours from smokers, a similar increased recruitment of β-arr1 to E2F-regulated promoters was found. Combined, these studies highlight an important role for the β-arr-Src scaffold and downstream signalling processes in the progression of different types of tumour.

3.2 β-arr Scaffolds in the PTEN/PI3K/AKT Pathway

Activation of PI3K leads to increased phosphatidyl-3,4,5-trisphosphate (PIP3) levels. PIP3 recruits a number of proteins, including AKT, to the membrane, by binding their PH domains (Chalhoub and Baker 2009). Activated AKT phosphorylates a range of substrates, either activating or inhibiting them, and this leads to cell growth, survival and proliferation. The IGF-1 receptor (IGF-1R) stimulates PI3K activity. IGF-1R signalling is important in various types of human cancers. A role for this receptor has been demonstrated in the transformation of cells, cancer cell proliferation and metastasis. When re-expressed in β-arr1−/− β-arr2−/− MEFs, β-arr1 was shown to activate PI3K downstream of the IGF-1 receptor and this occurred independently of the tyrosine kinase activity of the IGF-1R, which is the classical IGF-1 stimulated pathway (Povsic et al. 2003). The β-arr1-mediated activation of PI3K resulted in phosphorylation of AKT and increased protection from apoptosis. Another study demonstrated that β-arr2 can scaffold AKT and Src downstream of the insulin receptor leading to AKT activation (Luan et al. 2009). β-arrs have also been shown to activate AKT downstream of several GPCRs including PAR-1 (Goel et al. 2002) and the Ghrelin receptor (Lodeiro et al. 2009). In human microvascular endothelial cells, overexpression of β-arr1 caused increased matrix metalloproteinase (MMP9) activity and small blood vessel formation in a PI3K-dependent manner (Zou et al. 2008). This effect on MMP9 activity plays an important role in vivo by providing a suitable microenvironment for tumour progression in β-arr1 transgenic mice (Zou et al. 2008).

In contrast to the positive regulatory roles described above, β-arrs can also inhibit the PI3K/AKT pathway through different mechanisms. Both β-arr1 and β-arr2 have been shown to directly inhibit PI3K activity downstream of the PAR-2 receptor (Wang et al. 2007). β-arrs can also recruit phosphatases to AKT to "turn off" signalling. β-arr2 recruits PP2A to AKT, downstream of the dopamine D2 receptor and this results in dephosphorylation and inactivation of AKT (Beaulieu et al. 2005). In a similar fashion, β-arr1 has been shown to scaffold the pleckstrin homology domain leucine-rich repeats protein phosphatase (PHLPP) 2 with AKT (Crotty et al. 2013). Another point of impact of β-arrs in the pathway lies in their capacity to regulate the tumour suppressor PTEN (Lima-Fernandes et al. 2011). PTEN is a lipid phosphatase that opposes the action of PI3K by dephosphorylating PIP3 at position D3 to generate PIP2 (Chalhoub and Baker 2009). Both β-arrs directly bind PTEN and stimulate its activity in vitro

(Lima-Fernandes et al. 2011). Downstream of RhoA/ROCK signalling, β-arrs increase PTEN activity and thus inhibit AKT activation leading to reduced cell proliferation. In addition, β-arrs can also control lipid-independent functions of PTEN. The C2 membrane-binding domain of PTEN inhibits glioma cell migration, independently of its lipid phosphatase activity. Both β-arrs were found to release this brake on cell migration via the interaction with the C2 domain of PTEN (Lima-Fernandes et al. 2011). They thus modulate distinct functional outputs of PTEN signalling to differentially regulate cell proliferation and migration. β-arrs therefore have multiple points of impact along the PTEN/PI3K/AKT pathway and can play both negative and positive regulatory roles in this signalling axis, depending on upstream inputs and biological contexts.

3.3 β-arr Scaffolds Controlling Mdm2/p53

β-arrs can bind the oncoprotein Mdm2, an E3 ubiquitin ligase and major negative regulator of tumour suppressor p53 (Shenoy et al. 2001). Mdm2 is localised predominantly in the nucleus, where it ubiquitinates p53, targeting it for destruction and thus suppressing p53-mediated apoptosis. Due to its active nuclear export, β-arr2 can bind and redirect Mdm2 from the nucleus to the cytoplasm, thus relieving the degradation of p53 and increasing p53-dependent transcription and apoptosis (Scott et al. 2002; Wang et al. 2003a, b). This capacity is absent in β-arr1 due to lack of a functional NES. The lack of a NES in β-arr1 is due to a single amino acid difference (Gln394 instead of Leu394 found in human β-arr2). Mutation from Gln to Leu in β-arr1 results in a "gain-of-function" NES and this modified β-arr1 can redirect Mdm2 from the nucleus to the cytoplasm (Wang et al. 2003a; Song et al. 2006). Both β-arrs can form oligomers (Milano et al. 2006; Boularan et al. 2007) (see chapter "Self-association of Arrestin Family Members"), and the oligomerisation of β-arr2 was also shown to be required for the cytoplasmic sequestration effect on Mdm2 and subsequent increased p53-dependent anti-proliferative effects in NSCLC cells (Boularan et al. 2007). In contrast, following chronic stimulation of the β2AR, β-arr1 activates AKT, which results in the phosphorylation and activation of Mdm2 (Hara et al. 2011). Nuclear β-arr1 then scaffolds Mdm2 to p53, facilitating the ubiquitination and degradation of p53, which compromises genome integrity. Therefore β-arr1 and β-arr2 can play opposing roles in the regulation of Mdm2/p53 with β-arr1 promoting p53 degradation and β-arr2 increasing p53 stability. β-arrs can also recruit Mdm2 to other targets with relevance to cancer. For example, both β-arrs can target Mdm2 to the IGF-1R to promote its ubiquitination and downregulation (Girnita et al. 2005). β-arr2 can also bring Mdm2 and the androgen receptor (AR) into close proximity promoting AR ubiquitination and degradation (Lakshmikanthan et al. 2009). β-arr2 therefore acts as a corepressor of AR in agreement with an inverse correlation between β-arr2 and AR in prostate cancer tissue (see Sect. 2.2).

3.4 β-arr Scaffolds Controlling β-Catenin Signalling

β-arrs are also implicated in Wnt/β-catenin signalling both in cellular systems and in vivo (Kovacs et al. 2009; Schulte et al. 2010). Wnts are secreted glycoproteins that act through seven-transmembrane spanning receptors of the Frizzled (FZD) family. In the absence of Wnt ligand the Axin–APC scaffold promotes GSK3β phosphorylation of β-catenin, inducing its degradation. Binding of Wnt ligand to FZD, and a low-density lipoprotein-related protein 5/6 (LRP5/6) co-receptor, causes activation of Dishevelled (DVL) proteins, with subsequent inhibition of the Axin/GSK3β complex, resulting in stabilisation of β-catenin. β-catenin can then translocate into the nucleus, where it binds TCF/LEF transcription factors and promotes transcriptional activation.

The first evidence for the involvement of β-arrs in the β-catenin pathway came from a study demonstrating that β-arr1 interacted strongly with phosphorylated DVL1 and DVL2 (Chen et al. 2001). Co-expression of β-arr1 with either DVL1 or DVL2 led to a synergistic activation of LEF-mediated transcription. Subsequent studies showed that β-arr2 interacts with phosphorylated DVL and axin following Wnt3A stimulation of MEFs, demonstrating that β-arrs play a functional role in the inhibition of the β-catenin destruction complex (Bryja et al. 2007). Further support that the β-arrs play an important role in the regulation of the β-catenin destruction complex comes from studies investigating the link between the ETAR and β-catenin signalling in ovarian cancer cell invasion and metastasis (Rosano et al. 2009). As mentioned above in Sect. 2.2, ETAR and β-arr1 are co-expressed in advanced ovarian tumours (Rosano et al. 2009). Downstream of stimulated ETAR, β-arr1 activates Src, which promotes transactivation of the EGFR and subsequent phosphorylation of β-catenin. This phosphorylation enhances binding of β-catenin to TCF. In a parallel pathway, β-arr bound to axin, contributing to inactivation of GSK3β and β-catenin stabilisation. This β-arr-mediated regulation of β-catenin was shown to contribute to metastasis in ovarian cancer xenografts (Rosano et al. 2009). Blockade of the ETAR with a specific ETAR antagonist abrogated the effects of β-arr on the β-catenin pathway. A subsequent study from the same group showed that β-arr1 can translocate into the nucleus where it directly binds β-catenin and enhances β-catenin nuclear accumulation (Rosano et al. 2012). The β-arr1–β-catenin interaction augments β-catenin target gene expression required for cell migration, invasion and epithelial-to-mesenchymal transition. It does this by promoting HDAC1 dissociation and the recruitment of p300 acetyltransferase to the gene promoters, followed by enhanced H3 and H4 histone acetylation and gene transcription. In ovarian cancer tissue β-arr1–β-catenin complexes were also found enriched at β-catenin target promoters, compared to non-tumoural ovarian tissue, underscoring the significance of this interaction in tumour progression (Rosano et al. 2012).

Finally, two studies using murine models demonstrated that β-arr2 mediates the initiation and progression of tumourigenesis (Bonnans et al. 2012; Fereshteh et al. 2012). The mutation of the tumour suppressor gene *APC* is the earliest genetic event in colorectal tumours leading to activation of Wnt signalling. In the first

study, Bonnans et al. crossed $Apc^{\Delta 14/+}$ mice, a model of human intestinal carcinogenesis, with β-arr2$^{-/-}$ mice and observed that $Apc^{\Delta 14/+}$β-arr2$^{-/-}$ mice displayed significantly less tumours than control littermates. The β-arr2-dependent tumours showed increased expression of genes involved in Wnt signalling, cell adhesion, migration and extracellular matrix remodelling. In vitro studies indicate that β-arr2 is acting upstream or at the levels of the APC/GSK/Axin complex (Bonnans et al. 2012). In the second study, Fereshteh et al. also showed that β-arr2 acted upstream of β-catenin to promote its activation. This signalling effect of β-arr2 was implicated in the initiation and progression of myeloid leukaemia through the self-renewal capacity of the cancer stem cell population (Fereshteh et al. 2012). These two studies therefore elegantly demonstrate that β-arr2 is required for tumour initiation and progression via aberrant Wnt signalling.

From the above studies, it is clear that β-arrs can either act as drivers or as brakes on cancer pathways, depending on the associated signalling context and environmental cues. Receptor agonists, antagonists, biased agonists or small molecules that inhibit specific β-arr-partner interactions might thus be of use here to alter β-arr-mediated signalling and cancer biology outputs. In addition, as changes in β-arr expression and localisation are associated with cancer progression, strategies to intervene to change β-arr expression levels and localisation may also provide a basis for the development of potential therapeutics.

4 Discussion

Considering their important roles as key GPCR regulators and multifunctional signalling scaffolds, it is not surprising that β-arr levels are tightly regulated. The highest levels of β-arrs are found in the brain and spleen (Attramadal et al. 1992; Sterne-Marr et al. 1993) and their expression varies depending on the tissue, cell type and biological context. For example, expression of β-arr1 in foetal rat brain is selectively increased during neural differentiation (Gurevich et al. 2002, 2004) and some extreme differences in β-arr1 versus β-arr2 levels have been reported in the adult where only β-arr1 is detected in cells of the respiratory epithelium, while only β-arr2 is observed in olfactory receptor neurons (Dawson et al. 1993; Gurevich and Gurevich 2006). In addition to different expression levels, β-arr1 and β-arr2 also display some distinct properties in terms of their affinity for GPCRs, subcellular localisation, interacting partners and signalling (Scott et al. 2002; Song et al. 2006; DeWire et al. 2007; Moore et al. 2007). Thus changes in the expression of a single or both subtypes of β-arrs may have important physiological and pathological consequences. Indeed, in addition to cancer, the expression of β-arr1 and/or β-arr2 is altered in many human diseases ranging from neurodegenerative diseases (Bychkov et al. 2008; Thathiah et al. 2013) to coronary heart disease (Archacki et al. 2003) and type II diabetes (Luan et al. 2009). However, the mechanisms that control β-arr expression and stability remain poorly understood. Further studies are needed to address this critical question and some of the directions they may take are detailed below.

Many tumours harbour chromosomal deletions and/or amplifications that lead to dysregulation of signalling pathways. ARRB1 and ARRB2, the genes encoding β-arr1 and β-arr2, are located on separate chromosomes (Gurevich and Gurevich 2006) and they are found in regions known to undergo this type of modification. ARRB1 maps to chromosome locus 11q13 also containing the CCND1 gene (encoding cyclin D1), which is amplified in breast cancer and this is associated with reduced patient survival. Lundgren et al. (see Sect. 2.1) report an inverse correlation between β-arr1 expression and CCND1 amplification, which is known to be associated with the deletion of neighbouring sequences (Lundgren et al. 2011). ARRB2 is localised to chromosomal locus 17p13, close to the p53 gene. It is amplified or deleted in multiple cancers including breast cancer, NSCLC and leukaemia (Chuensumran et al. 2007). Although not reported, ARRB2 may therefore be affected by the genetic events occurring at this locus.

The regulation of β-arr gene expression is also an obvious mechanism regulating β-arr levels that has remained almost completely unexplored. β-arr1 gene expression is increased in response to stimuli that raise intracellular cAMP including forskolin, cholera toxin and isoproterenol (Parruti et al. 1993). Interestingly, elevated β-arr2 expression has been observed in samples from cystic fibrosis patients and in cystic fibrosis cell models. Elevated cAMP levels, secondary to the loss of the cystic fibrosis transmembrane conductance channel, promote the increase in β-arr2 concentration (Manson et al. 2008). Despite these intriguing results the mechanisms responsible for the regulation of β-arrs by cAMP are still unknown. A recent report demonstrated that both β-arr1 and β-arr2 gene expression is regulated by glucocorticoids (Oakley et al. 2012). Glucocorticoids enhance the expression of β-arr1 and repress the expression of β-arr2 via the recruitment of ligand-activated glucocorticoid receptors to conserved and functional glucocorticoid response elements (GREs) contained within introns of the β-arr genes. While intron 1 in the β-arr1 gene contains a classical GRE that functions as a glucocorticoid-dependent enhancer, intron 11 in the β-arr2 gene contains an inverted repeat negative GRE (nGRE) that acts as a glucocorticoid-dependent repressor, explaining these differential effects on β-arr1 and β-arr2 expression. Highlighting the functional significance of these alterations the authors demonstrated that the increase in β-arr1 levels resulted in decreased G protein coupling and increased β-arr1-dependent MAPK activation following PAR-1 activation in NSCLC cells. These results showing that regulated changes in β-arr gene expression translate into a switch of cell surface receptor signalling profile attest to the importance of this regulatory mechanism and have implications for GPCR-targeting drugs. They also highlight the need for further studies to identify other mechanisms that may regulate β-arr gene expression.

In addition to mechanisms that target the genes for β-arrs through chromosomal modification or transcription, changes in β-arr levels can also result from post-translational modification such as ubiquitination. It was reported that chronic insulin treatment leads to ubiquitination and proteasome-mediated degradation of β-arr1 (Dalle et al. 2002). This results in increased β2AR–Gs coupling and impaired β-arr1-mediated ERK activation following either IGF-1R or GPCR stimulation

(Dalle et al. 2002; Hupfeld et al. 2003). Another example of changes in β-arr levels through post-translational control comes from a study showing that antidepressants can induce β-arr2 ubiquitination and degradation in rat glioma cells (Golan et al. 2010). Interestingly, decreased β-arr1 protein has been reported in mononuclear leucocytes of depressed patients and correlated with the severity of the disease. The low level of β-arr1 is alleviated by antidepressant treatment; however, the mechanisms responsible for these changes are still unknown (Matuzany-Ruban et al. 2005).

As well as modifying β-arr expression levels, modulating subcellular localisation using therapeutic agents may also be of use in altering β-arr-dependent signalling. Both β-arr1 and 2 can be actively imported into the nucleus. β-arr2 is rapidly excluded due to the presence of an NES that is absent in β-arr1 (Scott et al. 2002; Wang et al. 2003a). β-arr1 accumulates in the nucleus following receptor stimulation, where it plays roles in transcriptional control (Kang et al. 2005; Ma and Pei 2007). Enhanced nuclear β-arr1 accumulation and associated HIF-1-dependent transcriptional regulation were also documented in a breast cancer cell system during hypoxia (Shenoy et al. 2012). Interestingly, treatment of the breast cancer cells with either thalidomide or imatinib mesylate, which can both suppress angiogenesis, promoted nuclear exclusion of β-arr1–HIF-1 complexes to perinuclear compartments and this was linked to reduced HIF1-mediated transcription. This study therefore provides proof of principle that agents capable of changing β-arr subcellular localisation can modify β-arr-mediated effects in cancer signalling pathways.

To conclude, changes in β-arr expression and localisation are associated with cancer progression. Further studies investigating changes in β-arr levels/localisation that occur during tumourigenesis will help to increase our understanding of how β-arr-dependent signalling processes may influence cancer-associated pathways and tumour progression. In addition to drugs that target receptors or protein–protein interactions to alter β-arr-mediated signalling, uncovering the mechanisms that regulate β-arr expression and localisation may also provide an alternative approach to identifying possibilities towards therapeutic intervention.

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β-Arrestins: Regulatory Role and Therapeutic Potential in Opioid and Cannabinoid Receptor-Mediated Analgesia

Kirsten M. Raehal and Laura M. Bohn

Contents

Abstract Pain is a complex disorder with neurochemical and psychological components contributing to the severity, the persistence, and the difficulty in adequately treating the condition. Opioid and cannabinoids are two classes of analgesics that have been used to treat pain for centuries and are arguably the oldest of "pharmacological" interventions used by man. Unfortunately, they also produce several adverse side effects that can complicate pain management. Opioids and cannabinoids act at G protein-coupled receptors (GPCRs), and much of their effects are mediated by the mu-opioid receptor (MOR) and cannabinoid CB1 receptor (CB1R), respectively. These receptors couple to intracellular second messengers and regulatory proteins to impart their biological effects. In this chapter, we review the role of the intracellular regulatory proteins, β-arrestins, in modulating MOR and CB1R and how they influence the analgesic and side-effect profiles of opioid and

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cannabinoid drugs in vivo. This review of the literature suggests that the development of opioid and cannabinoid agonists that bias MOR and CB1R toward G protein signaling cascades and away from β-arrestin interactions may provide a novel mechanism by which to produce analgesia with less severe adverse effects.

Keywords Opioid • Cannabinoid • Analgesia • Pain • Mu-opioid receptor • Cannabinoid CB1 receptor • Arrestin • Tolerance • Antinociception

1 Introduction

Pain is a complex disorder with neurochemical and psychological components contributing to the severity, the persistence, and the difficulty in adequately treating the condition. Although there are several different types of pharmaceutical drugs approved for the treatment of moderate to severe pain, it has been well documented that patients suffering from protracted persistent pain, especially those with cancer or neuropathic pain, often do not receive adequate relief from currently available analgesics (Brennan et al. 2007). Opioid and cannabinoids are two classes of analgesics that have been used to treat pain for centuries and are arguably the oldest of "pharmacological" interventions used by man. Unfortunately, they also produce several adverse side effects that can complicate pain management. Therefore, there remains a significant need to develop therapeutics with improved analgesic efficacy and reduced adverse effects. Since their discovery in the early 1990s, β-arrestins have proven to be important regulators of G protein-coupled receptors (GPCRs). Opioid and cannabinoids act at G protein-coupled receptors (GPCRs), and much of their effects are mediated by the mu-opioid receptor (MOR) and cannabinoid CB1 receptor (CB1R), respectively. These receptors couple to intracellular second messengers and regulatory proteins to impart their biological effects and β-arrestins may represent a means to fine-tune analgesic responses mediated by these receptors. In this chapter, we review studies that explore how β-arrestins impact opioid and cannabinoid drug responsiveness at the mu-opioid receptor (MOR) and cannabinoid CB1 receptor (CB1R) in vitro and in vivo with regard to how they influence the degree of analgesia and the side-effect profile of analgesic drugs.

2 Opioid and Cannabinoid Receptor Pharmacology

Opioids and cannabinoids produce their pharmacological effects through activation of GPCRs. There are four distinct genes coding for opioid receptors: the mu-, kappa-, and delta-opioid receptors (MOR, KOR, and DOR, respectively) and the

opioid-like receptor1 [ORL-1 or the nociceptin receptor (NOP)] (Cox 2012; Pasternak 2013). The generation of genetic knockout mice has demonstrated that the majority of clinically used opioids including morphine produce their pharmacological effects primarily by activating the MOR (Matthes et al. 1996; Sora et al. 1997; Roy et al. 1998; Kieffer 1999; Kieffer and Gaveriaux-Ruff 2002). The MOR is widely distributed and expressed in neurons in the brain, spinal cord, and the periphery (Gutstein and Akil 2001). Two major types of cannabinoid receptors have been identified: cannabinoid subtype 1 (CB1R) and cannabinoid subtype 2 (CB2R). While there is evidence demonstrating a modulation of pain responses by actions at CB2 receptors (Jaggar et al. 1998; Malan et al. 2001; Sokal et al. 2003; Elmes et al. 2004; Hohmann et al. 2004; Ibrahim et al. 2006; LaBuda et al. 2005), CB1 receptors (CB1R) in the central nervous system play the most pronounced role in mediating the analgesic, motor, and psychoactive effects of cannabinoids (Zimmer et al. 1999; Kelly and Chapman 2001; Hohmann et al. 2005; Pertwee 2005; Suplita et al. 2006; Dziaduleqicz et al. 2007). CB1R are widely expressed in the central and peripheral nervous systems (SvÍzenská et al. 2008).

The clinically observed effects produced by opioid and cannabinoid analgesics can be determined by how effectively the MOR and CB1R signal at the cellular level. There is a rich literature describing MOR and CB1R signaling pathways that lead to antinociceptive responses (for reviews, see Williams et al. 2013; Raehal et al. 2011; Smith et al. 2010) (Fig. 1). Upon activation, both receptors couple predominantly to $G\alpha_{i/o}$ proteins (Law et al. 2000; Howlett et al. 2002). In descending pain processing pathways, MOR and CB1R coupling to inhibitory heterotrimeric G proteins leads to a decrease in calcium influx resulting in decreased GABA transmission. The decrease in GABA release promotes disinhibition of the OFF nociceptive neurons and direct inhibition of ON cells, resulting in antinociception (for reviews, see Fields 2004; Rea et al. 2007; Palazzo et al. 2010; Fig. 1). In addition to inhibiting calcium flux, G proteins can modulate the activity of several different second messengers and cellular effectors, which may generate both short-term and long-term changes at the molecular and cellular levels resulting in diverse biological effects, including alternate paths to antinociception as well as neuroadaptations such as physical dependence.

Receptor signaling is determined not only by the activation of G protein-mediated signaling cascades, but also by several regulatory mechanisms including receptor desensitization, internalization, resensitization, and downregulation. Further, the signaling pathways and regulatory events can differ at a given receptor dependent on its cellular context. In other words, a receptor may signal via different G proteins when expressed in different neurons or may signal independently of G proteins altogether. Receptor regulation is essential as it aids in controlling the extent and duration of receptor signaling by preventing receptor overstimulation, promoting signal termination, and regulating cell surface expression of receptors. Although the signaling activity of MOR and CB1R can be regulated by several means, in vitro and in vivo studies have collectively shown that β-arrestins can substantially influence how the MOR and CB1 receptors respond to agonists.

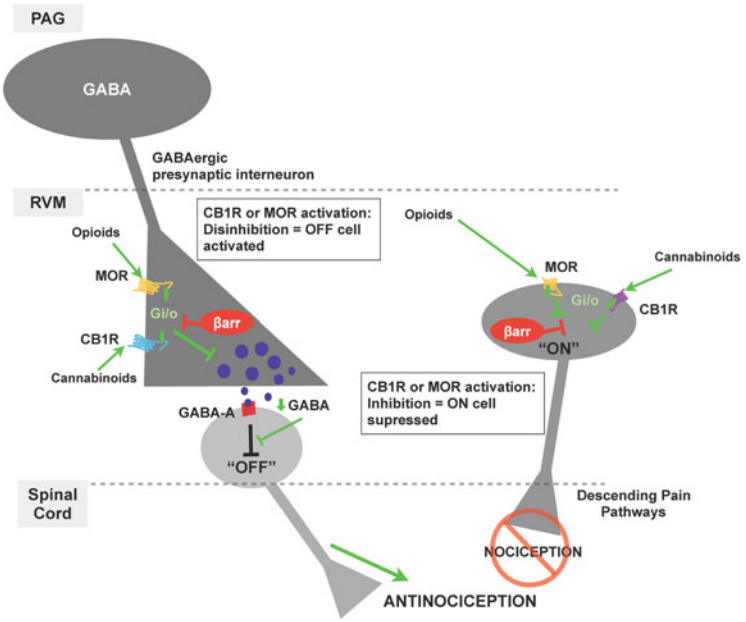

Fig. 1 β-arrestin modulation of antinociceptive responses mediated by MOR or CB1R expressed in descending nociceptive processing pathways. Activation of cannabinoid 1 receptors (CB1) or mu-opioid receptors (MOR) on GABAergic interneurons in the rostroventral medulla (RVM) decreases GABA release via mechanisms downstream of coupling to inhibitory Gαi/o proteins (activation of GIRKs, inhibition of voltage-dependent calcium channels, and inhibition of adenylyl cyclase pathways, among others). Normal GABA tone suppress "OFF" cells in the RVM by acting at GABA-A receptors, which raise action potentials. When GABA levels decrease, the tonic inhibition of "OFF" cells is relieved (i.e., disinhibition) and "OFF" cells signal to suppress pain perception in the spinal cord (descending pain perception regulation). In addition, activation of MOR or CB1Rs expressed on GABAergic "ON" cells in the rostral ventromedial medulla inhibits firing of these cells. Collectively, disinhibition of "OFF" cells and direct inhibition of "ON" cells produce analgesia; an effect can be measured using a thermal nociception tests. Since β-arrestins can inhibit the G protein signaling mechanisms utilized by CB1R and MOR, they can ultimately decrease antinociception as demonstrated in this simplified model of antinociception regulation (Fields 2004; Rea et al. 2007; Palazzo et al. 2010)

3 β-Arrestin-Mediated MOR and CB1R Regulatory Mechanisms

β-Arrestins belong to a family of four arrestin proteins (Gurevich and Gurevich 2003). Arrestins 1 and 4 are almost exclusively expressed in rod and cone cells in the visual system (Shinohara et al. 1987; Yamaki et al. 1987; Murakami et al. 1993; Craft et al. 1994) and are therefore referred to as "visual" arrestins. Arrestin 2 and arrestin 3 were first discovered for their ability to regulate the β_2-adrenergic receptor (Lohse et al. 1990) and, therefore, are also referred to as β-arrestin1 and β-arrestin2, respectively. Both β-arrestins are highly expressed in tissues throughout the central nervous system and periphery (Lohse et al. 1990; Attramadal et al. 1992; Gurevich

and Benovic 2000; Gurevich et al. 2002; Gainetdinov et al. 2004; Bychkov et al. 2012) and have been shown to regulate the activity of MOR and CB1 receptors.

β-Arrestins play a multifaceted role in regulating how GPCRs respond to agonist stimulation. One of the many ways in which β-arrestins regulate MOR and CB1R signaling is by promoting receptor desensitization. Following GPCR phosphorylation by GPCR kinases, β-arrestins bind to the phosphorylated MOR, which prevents further interactions between the receptor and G proteins even in the continued presence of agonist resulting in diminished G protein-mediated signaling (Zhang et al. 1998; Whistler and von Zastrow 1998; Kovoor et al. 1997, 1998; Bohn et al. 2000; Lowe et al. 2002; Eisinger et al. 2002; Celver et al. 2001, 2004; Qiu et al. 2003; Bailey et al. 2004, 2009; Koch et al. 2004; Dang et al. 2009, 2011). Studies of the CB1R also reveal an important role for β-arrestins in desensitizing the agonist-stimulated, phosphorylated CB1R (Sim et al. 1996; Jin et al. 1999; Daigle et al 2008; Nguyen et al. 2012).

In addition to disrupting G protein signaling cascades, β-arrestins can play a role in determining the fate of MOR and CB1 receptors, from the initiation of clathrin-dependent endocytosis (Jin et al. 1999; Whistler et al. 1999; Celver et al. 2004; Koch et al. 2004; Haberstock-Debic et al. 2005; Walwyn et al. 2006, 2007; Daigle et al. 2008; Groer et al. 2011; Patierno et al. 2011; Ahn et al. 2013) (see Chap. 9) to the recruitment of ubiquitin (E3) ligases involved in lysosomal-mediated receptor degradation (Groer et al. 2011; Henry et al. 2012; Malik et al. 2012) (see Chap. 10). The temporal and spatial scaffolding that β-arrestins impart in determining receptor interactions with signaling and regulatory elements can drive specific receptor signaling pathways as well as determine whether the receptor is resensitized or degraded. Studies of the MOR suggest that β-arrestin1 may play a role in ubiquitinating MOR and facilitating its dephosphorylation and potentially resensitization, while β-arrestin2 is prominently involved in desensitizing the receptor (Kovoor et al. 1997, 1998; Celver et al. 2001; Bohn et al. 2000; Dang et al. 2011; Groer et al. 2011). CB1 receptors have been shown to require β-arrestin2 for CP55940-induced internalization in HEK 293 cells, and the allosteric modulator, ORG27569, was shown to direct CB1 receptors to activating ERK cascades in a β-arrestin1-dependent manner (Ahn et al. 2013). It is becoming increasingly evident that there are several means by which β-arrestins can impact how the MOR and CB1R respond to agonists, which may have great bearing on how β-arrestins can mediate overall responsiveness to analgesic drugs.

4 β-Arrestin Regulation of MOR- and CB1R-Mediated Antinociception In Vivo

Due to the lack of selective inhibitors of arrestins at the turn of the century, the role of β-arrestins in opioid-induced analgesia was initially determined by assessing pain responses following morphine treatment of genetically modified mice lacking either β-arrestin1 or β-arrestin2 (Bohn et al. 1999, 2000, 2002). When administered

a single dose of morphine, β-arrestin1-KO mice respond normally compared to wild-type (WT) mice (Bohn et al. 2004); however, differences become readily apparent when β-arrestin2-KO mice are treated with morphine. β-Arrestin2-KO mice display enhanced and prolonged morphine-induced antinociception in paradigms that evaluate supra-spinal (hot plate) and spinal (tail flick) antinociceptive responses to a noxious thermal stimulus (Bohn et al. 1999, 2002, 2004; Raehal and Bohn 2011), suggesting that in the absence of the desensitizing effect of the β-arrestin2, MOR responsiveness is enhanced.

Similar responses to morphine observed in β-arrestin2-KO mice have been reported in normal mice and rats in which small interfering RNA (siRNA) or antisense oligonucleotides are used to knockdown β-arrestin2 in pain processing regions. siRNA inhibition of β-arrestin2 in the periaqueductal gray of mice enhances acute morphine-induced antinociception and delays the development of antinociceptive tolerance in the hot plate test (Li et al 2009). Morphine-induced antinociceptive tolerance in the tail flick test has also been shown to be significantly reduced in rats in which β-arrestin2 expression is knocked down in the spinal cord (Przewlocka et al. 2002; Yang et al. 2011). Moreover, the antinociceptive effects of morphine in the hot plate test are absent in rats overexpressing β-arrestin2 in the periaqueductal gray (Jiang et al. 2006). In contrast, siRNA knockdown of β-arrestin1 in rat periaqueductal gray had no effect on morphine-induced antinociception or the development of antinociceptive tolerance in the hot plate test (Li et al. 2009). This finding is consistent with cell culture studies wherein the morphine-bound MOR preferentially interacts with β-arrestin2 (Bohn et al. 2004; Groer et al. 2011).

Interestingly, not all MOR agonists produce enhanced antinociception in β-arrestin2-KO mice. Methadone, fentanyl, and etorphine produce a similar degree of antinociception as in their WT littermates in the hot plate test (Bohn et al. 2004; Raehal et al. 2011). In cell culture studies, these agonists promote both β-arrestin1 and β-arrestin2 interactions with the MOR, whereas morphine weakly and selectively recruits β-arrestin2 (Zhang et al. 2008; Whistler et al 1999; Bohn et al. 2004; Groer et al. 2007; McPherson et al 2010; Molinari et al. 2010). Therefore, morphine is a unique MOR agonist that selectively elicits β-arrestin2 regulation of the MOR; in its absence, β-arrestin1 may functionally substitute for the loss of β-arrestin2 in response to other MOR agonists.

Studies investigating the role of β-arrestins in CB1R-mediated analgesia and side effects have primarily been performed using β-arrestin2-KO mice. Upon acute treatment with the cannabinoid receptor agonists Δ^9-tetrahydrocannabinoid (THC) or CP55940, only THC produces an enhanced and prolonged response in the β-arrestin2-KO mice compared to vehicle controls as assessed by the hot plate (Breivogel et al. 2008) and tail flick (Breivogel et al. 2008; Nguyen et al 2012) tests. Similar to CP55940, other cannabinoid agonists including methanandamide and JWH-015 produce the same degree of antinociception in both WT and KO mice in the tail flick test (Breivogel et al. 2008). While it appears that the degree of MOR phosphorylation may be related to the propensity of an agonist to substitute β-arrestin1 for β-arrestin2 in desensitizing the MOR, the same correlations have

yet to be made for the CBR1. However, a recent report demonstrates that the two β-arrestins may have distinct roles in regulating the CB1R in an agonist-dependent manner, with β-arrestin1 mediating signaling events and β-arrestin2 determining internalization profiles in cell lines (Ahn et al. 2013).

5 β-Arrestin Regulation of Basal Nociceptive Resposes

An investigation of nociceptive behaviors in drug-naïve animals revealed that β-arrestin2-KO mice display longer basal warm water tail flick response latencies (Bohn et al. 2002; Breivogel et al. 2008; Lam et al. 2011; Nguyen et al. 2012), which can be blocked by the opioid antagonists naltrexone or naloxone (Bohn et al. 2002; Lam et al. 2011), but not the kappa- and delta-selective antagonists, norbinaltorphamine and naltrindole, respectively. This suggests that the prolonged response latencies are due to increased basal MOR activity or that the MOR is more sensitive to the presence of endogenous opioid peptides such as enkephalins and endomorphins. Alternatively, the deletion of β-arrestin2 may enhance the sensitivity of pro-nociceptive GPCRs. There is some evidence to support this idea as the transient receptor potential vanilloid 1 (TRPV1) channel, the transducer of thermal and chemical pain transmission, was found to be desensitized by β-arrestin2 (Por et al. 2012, 2013). Neurokinin receptors can also be regulated by β-arrestins (Schmidlin et al. 2003); however, no neurokinin receptor-mediated phenotypes have been reported in the β-arrestin2-KO mice. Further, while β-arrestin2-KO mice display prolonged basal antinociceptive response in assays with thermal endpoints such as the tail flick and Hargreaves tests (Bohn et al. 2002; Lam et al 2011), their basal responses in tests of mechanical stimulation using von Frey filaments are similar to their WT littermates (Lam et al. 2011), suggesting that β-arrestin2 may contribute to basal nociception thresholds in only certain types of pain pathways.

6 β-Arrestin Regulation of MOR and CB1R in Neurons

The manner in which β-arrestin2 regulates the MOR in distinct neuronal populations found in brain regions involved in modulating pain have been evaluated using β-arrestin2-KO mice. In periaqueductal gray and brain stem from KO mice, the MOR-selective agonist, DAMGO (D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin), produces a greater degree of receptor coupling to G protein compared to WT controls, suggesting that in the absence of the desensitizing β-arrestin2, the capacity for MOR to couple to G protein signaling cascades is enhanced (Bohn et al. 1999, 2000). Likewise, in the absence of β-arrestin2, DAMGO and morphine produce less inhibition of voltage-gated calcium channels in dorsal root ganglion neurons, which are involved in transmitting nociceptive information (Walwyn et al. 2007). Further,

under basal conditions, constitutive coupling to these channels is also enhanced (Walwyn et al. 2007).

The locus coeruleus (LC) is another brain region that contributes to the descending pain suppression pathway. In these neurons, acute enkephalin treatment produces the same degree of MOR-mediated desensitization of inward rectifying potassium channels in β-arrestin2-KO as WT controls (Dang et al. 2009). However, inhibition of ERK1/2 in β-arrestin2-KO mice alleviates desensitization, indicating that MOR desensitization in LC neurons is not solely dependent upon β-arrestin2 (Dang et al. 2009). Investigations of MOR resensitization profiles in LC neurons using the same system revealed that disruption of β-arrestin2-mediated receptor endocytosis enhances MOR resensitization rates (Dang et al. 2011). Further, WT but not βarrestin2-KO neurons treated chronically with morphine showed reduced rates of MOR resensitization, suggesting that β-arrestin2 determines MOR recycling profiles when morphine is used (Dang et al. 2011). Similarly, chronic morphine also impairs MOR recycling rates in WT but not KO. LC neurons isolated WT and β-arrestin2-KO mice (Quillinan et al 2011). However, this effect appears to be morphine dependent, as chronic methadone impairs MOR resensitization to the same extent in both genotypes (Quillinan et al. 2011).

Although the loss of β-arrestin2 has an overall effect of enhancing the antinociceptive efficacy of morphine, the manner by which β-arrestin2 regulates MOR to produce this response can vary depending on neuronal type as well as the agonist used. In the intact animal, it may also depend upon the pain state induced. While β-arrestin2 may play a substantial role in regulating MOR in vivo, it is not an exclusive modulator and likely part of a chain of events that ultimately determine receptor responsiveness. For example, treatment with the JNK inhibitors SP6 or BI78D3 or the PKC inhibitor bisindolylmaleimide VIII prior to morphine reverses the enhanced antinociceptive effect observed in β-arrestin2-KO mice to WT control levels in the tail flick test (Mittal et al. 2012). These studies suggest that activated JNK and/or PKC contribute to morphine-induced spinal antinociception. However, inhibition of JNK has no effect on fentanyl-induced antinociception in either genotype, indicating that the effect is ligand dependent (Mittal et al. 2012). In addition, elevated basal antinociceptive responses observed in β-arrestin2-KO mice are unaffected by JNK inhibition (Mittal et al. 2012). The lack of effect observed in the absence of drug with fentanyl may be explained by β-arrestin1 compensation in the β-arrestin2-KO mice or could depend on other regulatory proteins.

When examined for CB1R responsiveness, WT and β-arrestin2-KO mice were found to have similar levels of CB1R expression in the brain, although β-arrestin2-KO mice displayed greater antinociceptive responses to Δ^9-THC (Breivogel et al. 2008; Nguyen et al. 2012). When examined in spinal cord and across brain regions, the deletion of β-arrestin2 did not significantly impact upon CP55940-stimulated G protein coupling. However, after chronic treatment with Δ^9-THC, spinal cords from WT mice display significant decreases in CP55940-stimulated coupling to G protein and a downregulation of CB1R binding while these adaptations are not observed in β-arrestin2-KO mice (Nguyen et al. 2012).

7 β-Arrestin Mediation of Adverse Side Effects

While cannabinoids and opioids are effective pain relievers, they also produce a number of adverse side effects. Therefore, in addition to understanding how MOR and CB1R promote analgesia, there has also been significant interest in understanding the mechanisms that contribute to the side effects resulting from their activation. In the absence of β-arrestin2 morphine produces enhanced and prolonged analgesia, yet significantly less antinociceptive tolerance, physical dependence, constipation, and respiratory suppression (Bohn et al 1999, 2000, 2002; Raehal et al. 2005, 2011). The reduction in antinociceptive tolerance that develops following chronic morphine treatment appears to be due to a loss of β-arrestin2 desensitization of the MOR (Bohn et al. 2000). Following repeated Δ^9-THC administration, β-arrestin2-KO mice develop antinociceptive tolerance in the tail flick assay, yet to a lesser extent than that observed in the WT mice (Nguyen et al. 2012). This attenuated tolerance was shown to correlate with decreased CB1R desensitization of G protein coupling in cerebellum, caudal periaqueductal gray, and spinal cord (Nguyen et al. 2012)

In addition to tolerance, morphine produces physical dependence, constipation, and respiratory suppression in mice and in humans. When tested in the β-arrestin2-KO mice, morphine induces less constipation, an effect that appears to be due to altered β-arrestin2 regulation of MOR at the level of the colon (Raehal et al. 2005). Studies of colon preparations derived from the β-arrestin2-KO mice assayed in organ baths to assess morphine's inhibition of contractility suggest that morphine tolerance develops following repeated morphine treatment in the absence of β-arrestin2, while it does not develop in colon from WT mice (Kang et al. 2012).

At high doses, chronic morphine treatment produces physical dependence in the β-arrestin2-KO mice that is indistinguishable from the effects in the WT mice (Bohn et al. 2000; Raehal et al. 2011); however at lower doses of morphine infusion, β-arrestin2-KO mice are protected from the onset of dependence as evidenced by a decrease in the severity of the antagonist-precipitated withdrawal response (Raehal et al. 2011). It would seem that β-arrestin2 might facilitate the neuroadaptations that underlie the development of dependence but that it may not be exclusively required for the pathways to ensue.

Morphine-induced respiratory suppression is also decreased in the β-arrestin2-KO mice; however, like morphine-induced constipation and physical dependence, the mechanisms by which β-arrestin2 mediates these neuroadaptations remain unclear. If β-arrestin2 was primarily acting to desensitize MOR involved in mediating these side effects, one might predict that the severity of these side effects would be enhanced in β-arrestin2-KO mice. However, the side effects are less severe in the KO mice, suggesting that β-arrestin2 may be involved in mediating these responses in vivo. In cell culture studies β-arrestin2 has been shown to act as a scaffolding molecule to promote MOR signaling (Zhang et al. 2008). In neuronal preparations, β-arrestin2-mediated MOR signaling has been observed in dorsal root

ganglion neurons from β-arrestin2-KO mice, wherein DAMGO and morphine are less efficacious in inhibiting voltage-gated calcium currents (Walwyn et al. 2007). Fentanyl-induced MOR activation of ERK1/2 has also been shown to utilize β-arrestin2 in primary striatal neurons (Macey et al. 2006). However, β-arrestin2-mediated MOR signaling has not yet clearly been demonstrated in tissues associated with the onset of the side effects.

When treated with methadone or fentanyl, β-arrestin2-KO mice display the same degree of antinociceptive tolerance and physical dependence as WT controls (Raehal et al. 2011). Studies exploring the effects of other opioids on constipation and respiration have not been published, with the exception of loperamide. Loperamide (clinically used as the antidiarrheal Imodium®) is a peripherally restricted MOR agonist and does not delay colon transit times in the β-arrestin2-KO mice (Raehal et al. 2005). The β-arrestin-mediated effects on opioid constipation appear to be restricted to the periphery, as the genotype-dependent differences are not preserved when WT and β-arrestin2-KO are administered morphine by intracerebroventricular route (Bohn and Raehal 2006). Interestingly, loperamide, unlike morphine, leads to robust phosphorylation of the MOR in cell-based assays, which makes it more pharmacologically similar to methadone and fentanyl than morphine (unpublished observations). These findings suggest that the MOR in colon functions to delay transit via a β-arrestin2-dependent mechanism, although more studies must be undertaken to fully elucidate these mechanisms.

Cannabinoids also produce physical dependence as evidenced by signs of withdrawal upon cessation of drug taking, although to date, the role of β-arrestin2 has not been investigated in cannabinoid dependence. Cannabinoids also induce catalepsy and both genotypes display equivalent response profiles upon Δ^9-THC in the catalepsy ring test. However, the β-arrestin2-KO mice develop tolerance to a greater extent in this assay following chronic Δ^9-THC administration. It is not clear what mechanism underlies this behavioral adaption; however, the degree of CB1R desensitization of G protein coupling was greater in the cortex, globus pallidus, and substantia nigra of β-arrestin2-KO mice compared to the WT mice, suggesting that β-arrestin2-mediated desensitization of CB1R in these regions may play some role in mediating Δ^9-THC-induced catalepsy (Nguyen et al. 2012).

In mice, cannabinoids as well as opioids induce hypothermia. Morphine induces a greater drop in body temperature over time in β-arrestin2-KO mice compared to WT mice. β-arrestin2-KO display greater hypothermia in response to Δ^9-THC; however, tolerance to hypothermia develops in both genotypes (Breivogel et al. 2008; Nguyen et al. 2012). Interestingly, other agonists, including CP55940, methanandamide, and JWH-073, do not reveal a difference between genotypes in the hypothermia studies (Breivogel et al. 2008).

8 Therapeutic Potential of Biased Agonists in Pain Treatment

Biased agonists selectively engage one GPCR signaling pathway over another, such as coupling to a G protein over recruiting a β-arrestin (see Chap. 3). The pharmacological and genetic studies of rodents to date suggest that developing an opioid that does not recruit β-arrestin may represent a means to enhance antinociceptive efficacy while avoiding certain side effects. Presently, there have been a few reports of "biased" MOR agonists and their effects in vivo. One such compound herkinorin is a selective MOR agonist that does not recruit β-arrestin1 or β-arrestin2 in cell culture assays (Groer et al. 2007). In an inflammatory pain model in rat, herkinorin reduces formalin-induced flinching to the same degree as morphine when administered at the same dose (10 mg/kg, i.pl.), an effect that is reversed by the opioid antagonist naloxone (Lamb et al. 2012). Moreover, antinociceptive tolerance to herkinorin does not develop to repeated treatment over a 5-day period and it produces antinociception in morphine-tolerant rats (Lamb et al. 2012).

Another recently described compound, TRV130, has been reported to be a selective MOR agonist that produces robust G protein coupling but does not recruit β-arrestin2 (DeWire et al. 2013). In mice and rats, TRV130 is approximately five times more potent than morphine in tests (hot plate and tail flick) of thermal nociception. In a rat incisional pain model, TRV130 was as effective as morphine in treating tactile allodynia (DeWire et al. 2013). When given acutely, TRV130 also produces less constipation and respiratory suppression compared to mice treated with equi-efficacious doses of morphine (DeWire et al. 2013). The initial studies with these MOR "biased" agonists lend further support to the idea that developing an MOR agonist that does not engage β-arrestins but fully activates G protein signaling may provide a novel therapeutic avenue to improve pain treatment with opioids.

It also appears that biased agonism may represent a promising path for CB1R therapeutic development. The studies described herein indicate that β-arrestin2 regulates cannabinoid receptor-mediated thermal antinociception in a ligand- and tissue-dependent manner and developing cannabinoid agonists that do not promote receptor interactions with β-arrestins may improve the therapeutic profile of these types of analgesic drugs, while at the same time may promote desensitization for the catalepsy side effect. The continued development of biased agonists at each of these receptors and the study of their effect across species and physiologies will certainly broaden the degree of applicability of this merging concept.

9 Conclusions

It is apparent that β-arrestin-dependent regulation of MOR and CB1 receptors can profoundly impact how these receptors respond to their respective classes of analgesic drugs. Collectively, the studies discussed in this chapter show that

β-arrestin regulation of MOR and CB1 receptors is complex, so that the nature of how β-arrestins affect these receptors is influenced by the agonist acting at the receptor and the cellular environment in which the receptor is expressed. Moreover, opioid and CB1 agonists can direct receptors toward interactions with particular β-arrestins, which can affect the overall cellular signaling profile and biological response that is observed. The in vivo studies also suggest that not engaging β-arrestin interactions with the MOR or CB1 receptors can produce analgesia with less severe side effects. While initial studies in which selective and "biased" ligands at the MOR show promise in improving the overall therapeutic profile of opioid analgesics, extensive preclinical and clinical studies will be required to ultimately determine if a "biased" MOR and/or CB1R strategy will lead to the development of an analgesic with a wider safety margin that will improve the clinical treatment of pain.

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