Arrestin Interactions with G Protein-Coupled Receptors

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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.

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 β₂-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 β₂-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 β₂-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.
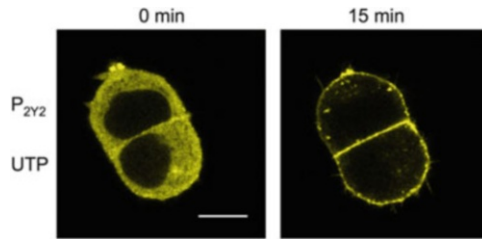
Fig. 1 β-Arrestin2-YFP translocation induced by stimulation of P2Y$_2$ receptor in transiently transfected HEK-293 cells. Images represent the same cells prior to (*left*) and 15 min after (*right*) stimulation with the agonist UTP (100 μM) [Data reproduced with permission from Hoffmann et al. (2008a)]

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
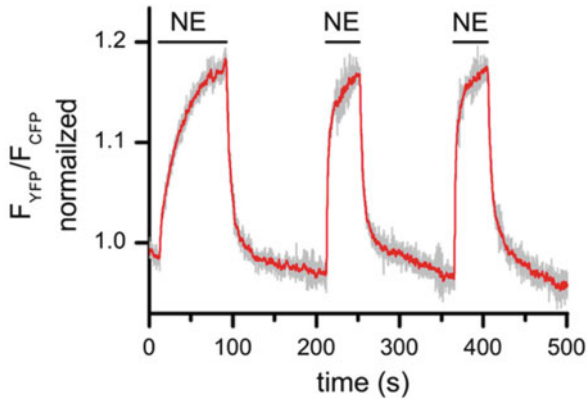
Fig. 2 Agonist-induced FRET between β_2-adrenergic receptor-YFP and β-arrestin2-CFP. Traces of FRET responses (F_{YFP}/F_{CFP}) to superfusion with the agonists in a single HEK cell transiently expressing β_2-adrenergic receptor-YFP and β-arrestin2-CFP. Agonist (300 μM norepinephrine) was present in 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
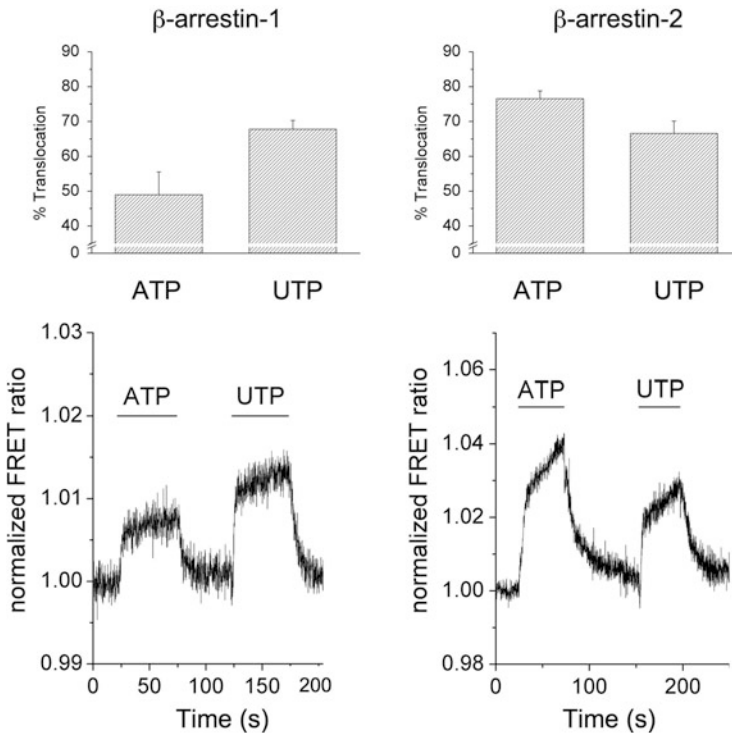
Fig. 3 Agonist (ATP vs. UTP)-selective interactions of P2Y$_2$ receptors with β-arrestin1 and β-arrestin2. The *top panels* show translocation to the cell surface, quantified from images as shown in Fig. 1, quantified as the loss of cytosolic fluorescence. The *bottom row* shows traces of FRET responses (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).
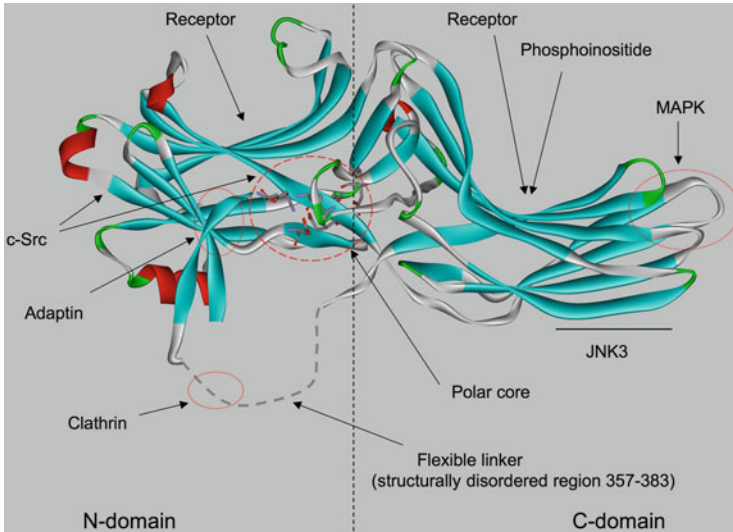
Fig. 4 Tentative assignment of protein interaction sites on β-arrestin. The polar core (D26, R169, K170, D290, D297, and R393) is indicated by the *dashed central circle*. Receptor binding has been shown to occur on multiple sites on the concave sides of β-arrestin. Proposed interaction sites for MAP 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
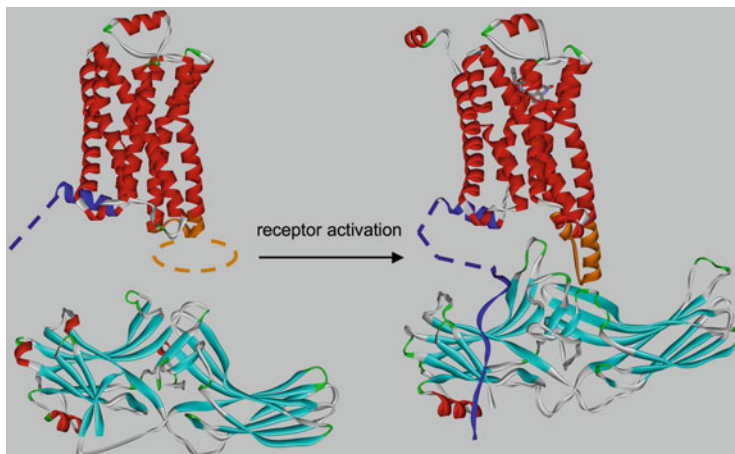
Fig. 5 Proposed scheme of agonist-mediated β-arrestin/receptor interaction. The *left side* of the scheme shows the crystal structures of the inactive β$_2$-adrenergic receptor bound to carazolol (PDB code 2RH1) and β-arrestin1 in its inactive state (PDB code 1G4M). The *dashed circle* and *line* indicate 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
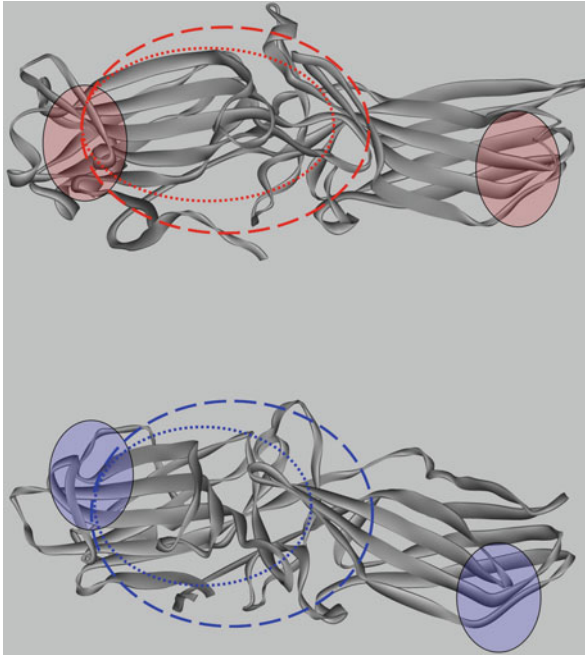
Fig. 6 Spatial requirements for arrestin/receptor interactions, indicating the receptor interaction sites on visual arrestin (*top*) and β-arrestin2 (*bottom*), together with the sizes of the cytosolic faces of rhodopsin (*top*) and the β$_2$-adrenergic receptor (*bottom*), respectively. The *top panel* 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
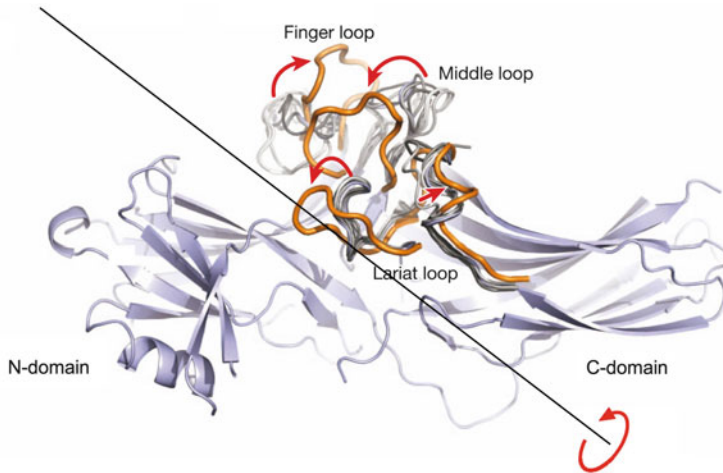
Fig. 7 Structural alterations occurring in β-arrestin1 upon binding of the C-terminal phosphopeptide of the vasopressin V_2 receptor. The figure was modified from Shukla et al. (2013). The *black line* represents the axis of the general 20° rotation of the C- versus the N-terminal domain that was observed 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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