REVIEW ARTICLE

Multifaceted roles of β-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling

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β-Arrestins are cytosolic proteins that bind to activated and phosphorylated G-protein-coupled receptors [7MSRs (seven-membrane-spanning receptors)] and uncouple them from G-protein-mediated second messenger signalling pathways. The binding of β-arrestins to 7MSRs also leads to new signals via activation of MAPKs (mitogen-activated protein kinases) such as JNK3 (c-Jun N-terminal kinase 3), ERK1/2 (extracellular-signal-regulated kinase 1/2) and p38 MAPKs. By binding to endocytic proteins [clathrin, AP2 (adapter protein 2), NSF (*N*-ethylmaleimide-sensitive fusion protein) and ARF6 (ADP-ribosylation factor 6)], β-arrestins also serve as adapters to link the receptors to the cellular trafficking machinery. Agonist-promoted ubiquitination of β-arrestins is a prerequisite for their role in receptor internalization, as well as a determinant of the differing trafficking patterns of distinct classes of receptors. Recently, β-arrestins have also been implicated as playing novel roles in cellular chemotaxis and apoptosis. By virtue of their ability to bind, in a stimulus-dependent fashion, to 7MSRs as well as to different classes of cellular proteins, β-arrestins serve as versatile adapter proteins that regulate the signalling and trafficking of the receptors.

Key words: adapter protein, β-arrestin, desensitization, endocytosis, G-protein-coupled receptor (GPCR), seven-membrane-spanning receptor (7MSR).

INTRODUCTION

The 7MSRs (seven-membrane-spanning receptors) are cell-surface receptors that respond to a variety of extracellular stimuli, including light, odour, chemoattractants, peptides, neurotransmitters, lipids and hormones. The 7MSR family includes at least 600 putative members represented in the human genome [1]. The 7MSRs were originally identified as receptors that couple to specific heterotrimeric G-proteins, thereby mediating G-protein-dependent signalling. Upon agonist binding, 7MSRs assume an active conformation and promote the dissociation of bound heterotrimeric G-proteins into Gα and Gβγ subunits. The activated G-protein subunits amplify and transduce signals within the cell by modulating the activity of effector molecules, such as adenylate cyclases, phospholipases and ion channels (Figure 1).

An important aspect of 7MSR regulation is the dampening of second messenger signalling that occurs in the continued presence of agonist. Phosphorylation events described below initiate this 'desensitization' of the receptors, leading to their uncoupling from their cognate G-proteins [2,3]. Two classes of serine/threonine kinases regulate the desensitization of the prototypic β_2AR (β_2-adrenergic receptor). The second-messenger-regulated kinases, PKA (protein kinase A) and PKC (protein kinase C), belong to the first group of enzymes, which can phosphorylate a receptor even in the absence of its agonist, resulting in 'heterologous desensitization'. [4]. However, the presence of an agonist-stimulated desensitization mechanism in cells that lack functional PKA (kin$^-$ S49 lymphoma cells) led to the discovery of GRKs [G-protein-coupled receptor (GPCR) kinases] [5]. The GRKs phosphorylate only those receptors that have been agonist-stimulated (i.e. activated) and hence mediate 'homologous desensitization'. However, GRK-mediated phosphorylation alone does not inhibit the receptor–G-protein interaction. Rather, the phosphorylated receptor recruits another molecule, β-arrestin, that prevents further G-protein binding (see below). To date, seven GRKs (GRK1–GRK7) have been identified [1]. GRK1 and GRK7, the kinases that phosphorylate opsins, are retinal enzymes. The one other enzyme that has restricted tissue distribution is GRK4, which is expressed mainly in the brain, kidney and testes. GRK2 [also known as βARK1 (β-adrenergic receptor kinase 1)], GRK3 (βARK2), GRK5 and GRK6 are ubiquitously distributed.

The GRKs consist of three distinct domains. The N-terminal region of GRK1–GRK7 is similar to an RGS (regulators of G-protein signalling) domain, and is termed the RH (RGS homology) domain. The function of RGS proteins is to inhibit the activity of Gα subunits by acting as GAPs (GTPase-activating proteins). The RH domain of GRK2 binds *in vitro* to the $G\alpha_q$ and $G\alpha_{11}$ proteins in the presence of aluminium fluoride (AlF_4^-), but does not induce GTPase activity. Other Gα proteins, such as $G\alpha_s$, $G\alpha_i$ and $G\alpha_{12/13}$, do not interact with the RH domain of

Abbreviations used: AP, adapter protein; β_2AR, β_2-adrenergic receptor; βARK, β-adrenergic receptor kinase; ARF, ADP-ribosylation factor; ARNO, ARF nucleotide binding site opener; ASK, apoptosis signal-regulating kinase; $AT_{1A}R$, AT_{1A} angiotensin receptor; Dvl, Dishevelled; ERK, extracellular-signal-regulated kinase; Fz4, Frizzled 4; GAP, GTPase-activating protein; GDS, GDP dissociation stimulator; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; IGF, insulin-like growth factor; JNK, c-Jun N-terminal kinase; JIP, Jnk-interacting protein; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEK, MAPK/ERK kinase; 7MSR, seven-membrane-spanning receptor; NSF, *N*-ethylmaleimide-sensitive fusion protein; PAR, proteinase-activated receptor; PDE, phosphodiesterase; PH domain, pleckstrin homology domain; PKA, protein kinase A; PKC, protein kinase C; RGS, regulators of G-protein signalling; RH domain, RGS homology domain; SAPK, stress-activated protein kinase; SDF, stromal cell-derived factor; SH3 domain, Src homology 3 domain; siRNA, small interfering RNA; Ub, ubiquitin; V_2R, V_2 vasopressin receptor.

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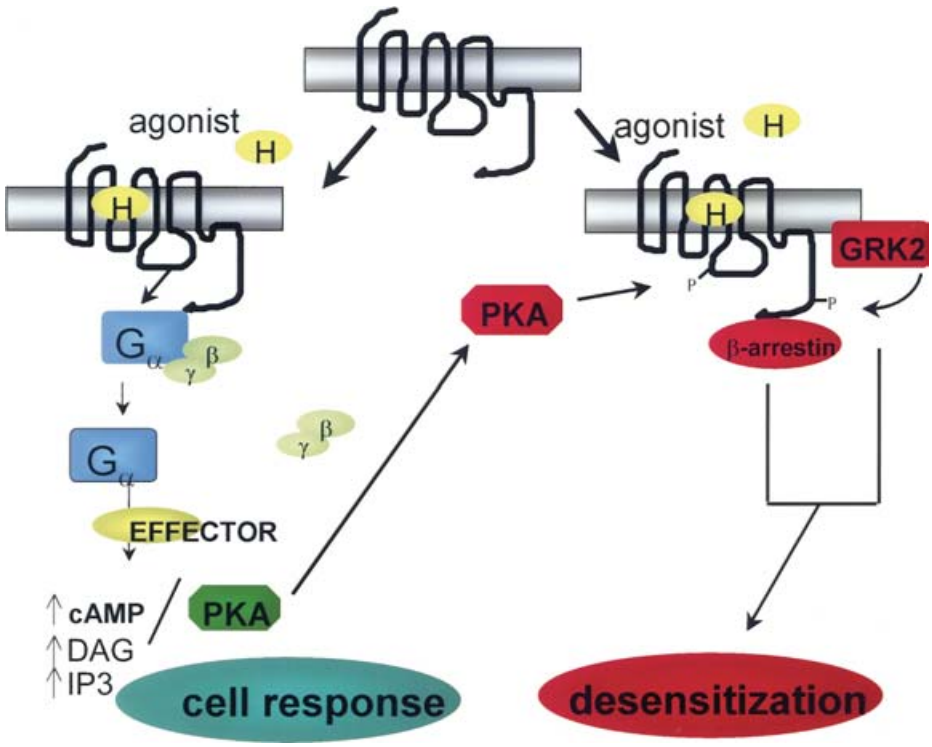

Figure 1 Classical role of β-arrestins: desensitization

When an agonist binds to a 7MSR, a transient high-affinity receptor–heterotrimeric G-protein complex is formed. GDP is released from the G-protein subunits and is replaced by GTP, leading to the dissociation of G-proteins into α and $\beta\gamma$ dimers. The G-protein subunits activate several effector molecules: $G\alpha_s$ activates adenylate cyclases; $G\alpha_q$ activates phospholipase C etc. In the case of the β_2AR, increased cAMP leads to activation of PKA, which phosphorylates the receptor as a feedback mechanism. The agonist-stimulated receptor is also a substrate for GRK-mediated phosphorylation that promotes β-arrestin binding. β-Arrestins prevent further G-protein coupling and G-protein-mediated second messenger signalling. DAG, diacylglycerol; IP3, Ins(1,4,5)P_3.

GRK2 [6]. The central catalytic domain of GRK is most similar to the AGC (PK**A**/PK**G**/PK**C**)-type kinases. The GRKs differ in their C-terminal region. GRK2 and GRK3 have a PH (pleckstrin homology) domain that binds to the $\beta\gamma$ subunits of G-proteins and PtdIns(4,5)P_2. The binding of prenylated G$\beta\gamma$ subunits and PtdIns(4,5)P_2 facilitates the membrane recruitment of GRK2 and GRK3, whereas direct palmitoylation of GRK4 and GRK6 or farnesylation of GRK1 and GRK7 are mostly responsible for the constitutive membrane association of these GRKs. Numerous regulatory mechanisms for the membrane association, activation and inactivation of the GRKs have been reported [7–13].

Phosphorylation-independent desensitization of receptors by GRKs has been reported for several 7MSRs ([14] and references cited therein). Studies on endothelin (ET_A and ET_B) receptors showed that, although both GRK2 and GRK5 could phosphorylate the receptors, GRK2 overexpression had greater effects to suppress phosphoinositide accumulation [15]. Additionally, overexpression of a mutant GRK2 (K220R; dominant negative) showed desensitizing effects similar to the wild type. These experiments indicated that receptor phosphorylation and receptor desensitization could be two independent outcomes of receptor–GRK interaction. In addition, co-immunoprecipitation assays showed that the endothelin receptors bound preferentially to GRK2 in an agonist-dependent manner, suggesting that the association of GRK with the receptor could play a major role in desensitization. Similarly, overexpression of GRK2-K220R can attenuate G_q signalling in the case of metabotropic glutamate receptor 1a [16].

Very recently, the crystal structure of a protein complex between bovine GRK2 and Gβ_1 and Gγ_2 subunits has been solved [17]. This atomic structure has greatly helped us to appreciate and understand the distinct roles of the three domains of the GRK enzyme. In the solved structure, the three domains of GRK2, namely the N-terminal RH domain, the central kinase domain and the C-terminal PH domain, are positioned at the three vertices of an equilateral triangle, separated by approx. 80 Å (where 1 Å $\equiv$ 0.1 nm; Figure 2). This arrangement allows the three domains of GRKs to position and interact with three distinct proteins simultaneously: the RH domain with a Gα subunit, the PH domain with G$\beta\gamma$ subunits and the kinase domain with the cytoplasmic regions of a 7MSR. This unique feature allows GRKs to phosphorylate receptors via the catalytic domain leading to desensitization events, while simultaneously sequestering already released Gα and G$\beta\gamma$ subunits, thereby preventing them from activating their effectors. This presents an extremely efficient dual mechanism for preventing downstream signalling via the G-proteins.

DISCOVERY OF β-ARRESTINS

The β-arrestins were discovered as a consequence of observations made in the course of purification of βARK (GRK2). Progressive purification of the enzyme from bovine brain was associated with a progressive decline in the ability of the kinase to 'desensitize' the β_2AR-mediated activation of Gα_s, assessed in a reconstituted system of purified proteins. An abundant retinal protein, originally

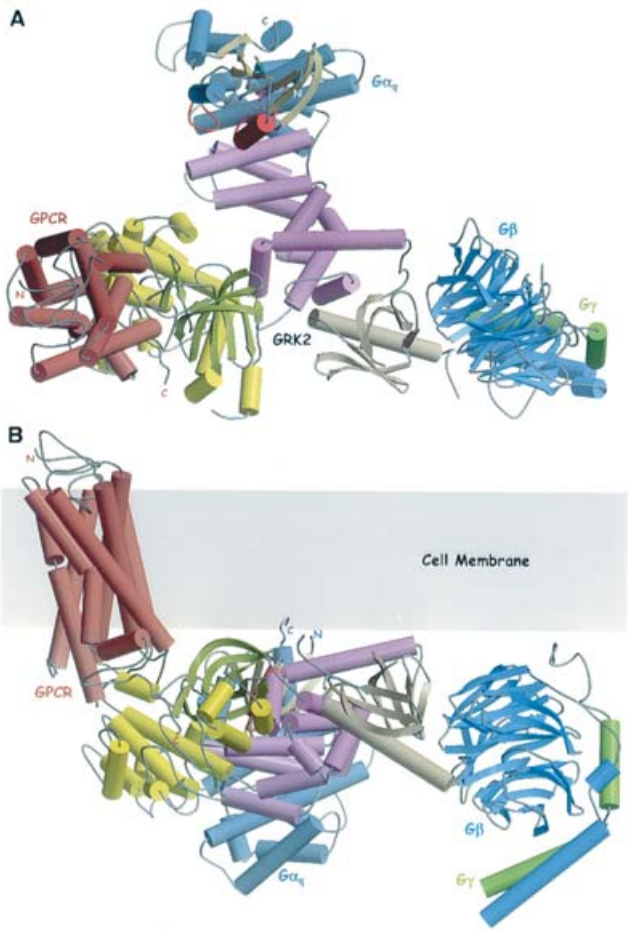

Figure 2 Simultaneous interaction of GRK with $G\alpha_q$, $G\beta\gamma$ and receptor subdomains

(**A**) A docking model based on the recent crystal structure of GRK–$G\beta\gamma$ along with the available models of $G\alpha_q$ and rhodopsin docked on the respective domains of GRK as seen from the cell membrane (receptor, maroon; $G\alpha_q$, dark blue with tan β strands). The switch regions of $G\alpha_q$ are indicated in red. The N- and C-termini of the receptor and of $G\alpha_q$ are indicated. (**B**) Side view of the complex, rotated 90° around the horizontal axis with respect to (**A**). The grey bar represents the cell membrane. Reprinted with permission from Lodowski, Pitcher, Capel, Lefkowitz and Tesmer (2003) Science **300**, 1256–1262. Copyright 2003 American Association for the Advancement of Science.

called S-antigen or 48K protein and subsequently renamed arrestin, had recently been found to act in concert with a retinal enzyme, rhodopsin kinase, to terminate light-activated rhodopsin-mediated signalling. When this protein was added back to the purified βARK, it largely restored its 'receptor-inactivating' ability. However, very high arrestin/G_s ratios were required compared with the rhodopsin system (≈200–300-fold) [18]. These findings suggested the presence of other arrestin-like proteins in non-retinal tissues that act in concert with the GRKs to desensitize the receptors. Subsequently, two isoforms of non-visual β-arrestins were cloned, namely β-arrestin1 (arrestin2) and β-arrestin2 (arrestin3) [19,20]. Both β-arrestin isoforms are ubiquitously distributed. These non-visual arrestin isoforms show marked specificity for 7MSRs other than rhodopsin. Subsequently cone arrestin, a fourth arrestin family member, was cloned [21].

Since the discovery of β-arrestins as proteins that desensitize receptor second-messenger signalling, several new roles and interacting proteins of β-arrestin have been identified (Figure 3, Table 1). Here we review these new realms of β-arrestin-mediated regulation of heptahelical receptor signalling.

RECEPTOR–β-ARRESTIN INTERACTION

Several early studies documented the paradigm that agonist stimulation of many 7MSRs leads to GRK-mediated phosphorylation followed by binding of β-arrestin to the phosphorylated receptor. The receptor–β-arrestin interaction has been demonstrated *in vitro* with purified proteins for the β_2AR, and by cellular co-immunoprecipitation for many receptors [22–27]. The development of GFP (green fluorescent protein)–β-arrestin fusion proteins has allowed visualization of the recruitment of cytosolic β-arrestin to receptors at the plasma membrane after agonist stimulation [28]. For most receptors, the determining factor for β-arrestin interaction is the phosphorylation status of the activated 7MSR. Thus impairment of receptor phosphorylation by mutagenesis of the key serine/threonine residues generally leads to diminished β-arrestin binding after stimulation of the m_2 muscarinic receptor, rhodopsin, the $AT_{1A}R$ (AT_{1A} angiotensin receptor) or the V_2R (V_2 vasopressin receptor) [26,29–31]. Furthermore, enhancement of receptor phosphorylation by overexpression of GRKs promotes the translocation and recruitment of GFP–β-arrestin to several receptors. The relevant phosphorylation sites are serine/threonine residues in the cytoplasmic C-terminal tail region and/or the third cytoplasmic loops of 7MSRs.

Although it has been demonstrated repeatedly that receptor phosphorylation generally precedes and is required for binding and activation of β-arrestin, certain exceptions do exist. Complex-formation between the human lutropin receptor and β-arrestins is independent of receptor phosphorylation and dependent on receptor activation [25]. Thus a mutant receptor that is not phosphorylated can still bind β-arrestin and internalize upon stimulation with human chorionic gonadotropin. The human lutropin receptor mutations $Leu^{457} \rightarrow Arg$, $Asp^{578} \rightarrow Tyr$ and $Asp^{578} \rightarrow His$ are found in individuals with Leydig cell hyperplasia and precocious puberty. These mutant receptors are constitutively activated and desensitized; hence addition of human chorionic gonadotropin does not lead to further cAMP accumulation. In fact, these mutant receptors can associate constitutively with β-arrestin in the absence of agonist stimulation and receptor phosphorylation [25]. Additionally, agonist-induced conformational changes themselves seem to be sufficient for receptor binding of certain mutant forms of β-arrestins, such as β-arrestin1^{R169E} and β-arrestin1^{1-383} [32]. In the case of these mutant β-arrestins, which are presumably in a state that mimics the activated conformation, the receptor–β-arrestin interaction appears to be independent of receptor phosphorylation.

CLASSICAL ROLES OF β-ARRESTIN: DESENSITIZATION

The binding of β-arrestin to GRK-phosphorylated receptors physically interdicts the coupling of the receptor to its cognate G-protein, thus achieving the cessation of G-protein-mediated signalling. The desensitization function of β-arrestin proteins has been characterized in several different ways [20,33,34]. Attramadal et al. [20] used purified recombinant β-arrestin1 and β-arrestin2 proteins from COS cells and tested their efficacy to blunt GTPase activity in an *in vitro* reconstituted β_2AR/G_s model system. Both β-arrestin1 and β-arrestin2 produced 80% inhibition of the measured GTPase activity stimulated by the β_2AR. Additionally, in cell lines with overexpression of β_2AR, transfection of β-arrestin leads to greater desensitization. Furthermore, recently developed β-arrestin siRNA (small interfering RNA)

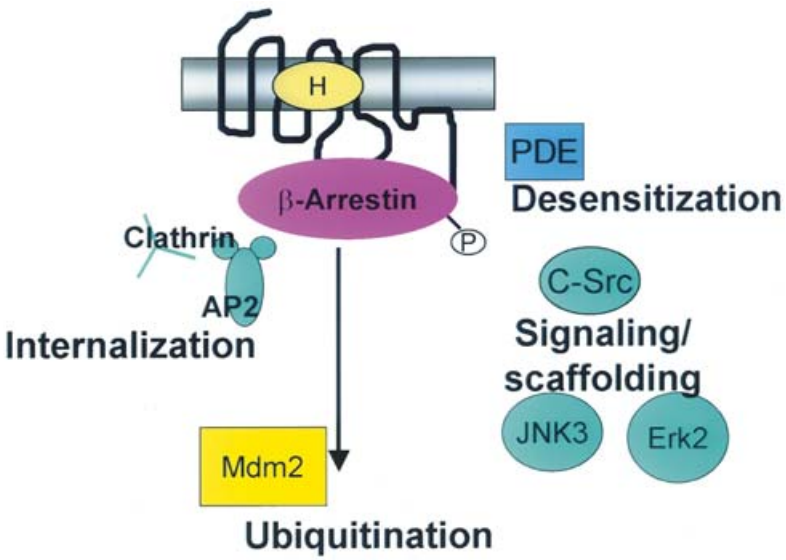

Figure 3 Expanded roles of β-arrestin

Several protein–protein interactions of β-arrestin with endocytic and signalling proteins are depicted. These novel interactions expand the roles of β-arrestin to receptor internalization, MAPK and other forms of signalling, and ubiquitination.

Table 1 Protein/lipid-interacting domains mapped on β-arrestin isoforms

Residues	Isoform/species	Binding partner	Reference
1–185	β-Arrestin1/rat	Src-SH1	[84]
1–185	β-Arrestin2/rat	ASK1	[92]
185–410	β-Arrestin2/rat	JNK3	[92]
1–185	β-Arrestin2/human	Mdm2	[111]
1–260	β-Arrestin2/rat	Mdm2	*
Pro^{91}, Pro^{121}	β-Arrestin1/rat	Src-SH3	[24]
Arg^{394}, Arg^{396}	β-Arrestin2/rat	B Adaptin-2	[54]
Leu-Ile-Glu-Phe	β-Arrestin1,2/bovine	Clathrin	[52]
Lys^{233}, Arg^{237}, Lys^{251}	β-Arrestin2/bovine	Phosphoinositides	[55]

* S. K. Shenoy and R. J. Lefkowitz, unpublished work.

methods have proven effective in demonstrating that reducing endogenous β-arrestin expression in HEK 293 cells results in greater cAMP accumulation upon stimulation of endogenous β_2ARs [35]. Similar effects on receptor desensitization were also observed on reducing endogenous β-arrestin expression by an antisense strategy [36]. In addition, desensitization of both the β_2AR and the AT_{1A}R is impaired in mouse embryonic fibroblast cell lines lacking both β-arrestins [37]. Single knockouts for β-arrestin1 or β-arrestin2 display modest impairment of both β_2AR and AT_{1A}R desensitization. However, in the case of PAR1 (proteinase-activated receptor 1), desensitization is mediated primarily by the β-arrestin1 isoform, since PAR1 is not desensitized in β-arrestin1 null mouse embryonic fibroblast cell lines, but is in β-arrestin2 null lines [38].

Stimulation of G_s-coupled receptors such as the β_2AR leads to the activation of adenylate cyclase and the production of cAMP. The increase in cellular cAMP levels results in turn in the activation of kinases such as PKA, which phosphorylate many substrates and lead to diverse cellular responses. PDEs (phosphodiesterases) are enzymes that degrade cAMP and regulate the levels of cAMP in the cell. Recent studies by Perry et al. [39] demonstrated that β-arrestins recruit the cAMP-degrading PDE4D enzymes to the activated β_2AR at the plasma membrane. PDE4D is not recruited to the membrane in cells that are null for β-arrestins, suggesting that β-arrestins specifically scaffold these enzymes at the membrane. Overexpression of a catalytically inactive PDE4D mutant (PDE4D5 D556A), which can bind β-arrestin and compete with endogenous PDE4D for membrane recruitment, leads to lower rates of degradation of cAMP at the membrane. β-Arrestins thus display dual regulation in desensitizing $G\alpha_s$-coupled receptors such as the β_2AR by simultaneously decreasing the rate of cAMP production (uncoupling the G-protein) while increasing cAMP degradation by recruiting and scaffolding cellular PDEs.

In order to characterize the physiological roles of β-arrestins *in vivo*, knockout mice models have been generated. Knocking out both β-arrestins results in embryonic lethality. Single knockouts are viable and do not display grossly abnormal phenotypes. However, significant differences were observed when 7MSRs were stimulated in these animals. For example, cardiac responses were hyperactive when β-arrestin1 null mice were challenged with isoprenaline (isoproterenol). β-Arrestin1 homozygous mutants were more sensitive to isoprenaline-stimulated increases in ejection fraction, consistent with a role of β-arrestin1 in βAR receptor desensitization [40]. Homozygous β-arrestin2 knockout mice showed prolonged and enhanced analgesia to morphine treatment due to an impairment in μ-opioid receptor desensitization [41]. Remarkably, β-arrestin2 null mice were completely unable to develop tolerance to the antinociceptive effects of morphine, and yet developed physical dependence on the drug similar to the wild-type controls [42]. These results suggest that the chemical phenomenon of opiate tolerance is due to a β-arrestin-dependent mechanism which is distinct from that causing physical dependence.

EXPANDED ROLES OF β-ARRESTINS: ENDOCYTOSIS

Subsequent to desensitization, receptors are removed from the cell surface by a process of internalization (also called endocytosis or sequestration). Sequestration does not seem to be necessary

for desensitization, but rather appears to be required for dephosphorylation and resensitization of activated receptors [43]. GRK-mediated phosphorylation and β-arrestin binding serve to promote receptor sequestration. Mutations that reduce agonist-dependent phosphorylation of the receptor result in impaired β-arrestin interaction and are poor internalization. For example, mutation of all the GRK phosphorylation sites impairs the sequestration of β_2AR [44]. Mutation of a highly conserved tyrosine residue (Y326A) in β_2AR diminishes both agonist-promoted phosphorylation and sequestration [45]. However, overexpression of βARK1 (GRK2) or β-arrestin and GRK2 together can rescue the internalization of the β_2AR-Y326A mutant [45,46].

Sequestration of 7MSRs is mediated by several mechanisms, including clathrin-coated vesicles, caveolae and uncoated vesicles [47]. The specific pathway utilized and the rate of internalization is characteristic of the particular receptor as well as the cell type in which it is expressed. In general, many 7MSRs interact with β-arrestins and are internalized via clathrin-coated pits. Initial appreciation of the role of β-arrestin in receptor internalization paralleled the finding that activated β_2ARs localized to clathrin-coated pits at the plasma membrane [48]. Ferguson et al. [49] reported that mutant β-arrestin proteins inhibited β_2AR internalization and that overexpression of β-arrestin could rescue sequestration-impaired β_2AR mutants. Goodman et al. [50] first demonstrated a distinct and direct interaction between β-arrestin1 or β-arrestin2 and clathrin by *in vitro* binding methods. β-Arrestins interact stoichiometrically and with high affinity with the clathrin heavy chain. The binding region maps to residues 89–100 on the clathrin terminal domain. Thus β-arrestins both desensitize agonist-activated receptors and, by binding to clathrin, promote their internalization via clathrin-coated pits. The β-arrestin–clathrin interaction is dependent upon a clathrin binding motif, Leu-Xaa-Glu/Asp, present near the C-terminus of non-visual arrestins [51,52]. Interestingly, β-arrestins also interact with the β_2 subunit of the clathrin–AP2 (adapter protein 2) complex, as demonstrated by yeast two-hybrid, cellular co-transfection and *in vitro* assays [53,54]. Two arginine residues (394 and 396) downstream of the clathrin binding region are important for the β-arrestin2–AP2 interaction. Mutation of these residues in β-arrestin does not affect binding to the β_2AR, but blocks targeting of receptor–β-arrestin complexes to clathrin-coated pits. Thus, by binding to both clathrin and AP2, β-arrestin serves as an important adapter to link the receptors to the internalization machinery.

Gaidarov et al. [55] found that β-arrestins display high-affinity phosphoinositide/phosphoinositol binding. By site-directed mutagenesis, three residues (Lys^{233}, Arg^{237} and Lys^{251}) of bovine β-arrestin2 were found to be essential for phosphoinositide binding. *In vitro* studies showed that a mutant in which these residues were mutated, which was defective in binding phosphoinositides, retained normal binding to clathrin and rhodopsin. However, the mutant was not recruited to clathrin-coated pits and failed to promote β_2AR internalization in COS cells.

An interaction between NSF (*N*-ethylmaleimide-sensitive fusion protein, an ATPase that is essential for many intracellular trafficking pathways) and β-arrestin1 has been shown by yeast two-hybrid and *in vitro* assays [56]. NSF also interacts with β-arrestin1 and β-arrestin2 in cellular co-immunoprecipitation assays. Functionally, overexpression of NSF in HEK 293 cells further enhances β-arrestin-mediated β_2AR internalization.

Another protein important for vesicular trafficking, the small GTP binding protein ARF6 (ADP-ribosylation factor 6), also binds β-arrestin upon β_2AR stimulation [57]. Activation of ARF6 requires replacement of GDP by GTP, and is facilitated by the GEF (guanine nucleotide exchange factor) activity of ARNO (ARF nucleotide binding site opener). Interestingly, ARNO binds constitutively to β-arrestin2, with the interaction being detectable even at endogenous levels of proteins. Both dominant-negative ARF6 (T27N, which mimics the GDP-bound form) and a constitutively active mutant ARF6 (Q67L; GTP-bound form) diminish β_2AR internalization. However, overexpression of ARNO, which can facilitate the conversion of GDP–ARF into GTP–ARF, enhances receptor internalization. The interaction of β-arrestin with both ARF6 and ARNO allows it to function as a receptor-regulated-scaffold-cum-'switch' to cause a transition of ARF6 from an inactive to an active form. Activated ARF6 proteins released from the complex are then available to facilitate receptor endocytosis.

Ral-GDS (GDP dissociation stimulator), a protein that catalyses GDP/GTP exchange on the small G-protein Ral, interacts with β-arrestin. Upon stimulation of the chemokine formyl-Met-Leu-Phe receptor, β-arrestin–Ral-GDS complexes are recruited to the membrane [58]. Dissociation of β-arrestin and Ral-GDS at the membrane results in its GEF activity, leading to activation of Ral and consequent cytoskeletal rearrangements culminating in chemotaxis and granule release.

Both β-arrestin1 and β-arrestin2 are cytosolic phosphoproteins. A serine residue (Ser^{412}) in β-arrestin1 is constitutively phosphorylated in cells [59]. Stimulation by isoprenaline leads to rapid dephosphorylation of β-arrestin, which is required for β-arrestin–clathrin interaction. The S412D mutant β-arrestin1, which mimics phospho-β-arrestin1, does not bind clathrin and acts as a dominant-negative for β_2AR sequestration. The dephosphorylation of β-arrestin1 appears to be transient, at least with respect to β_2AR stimulation. The isoprenaline-activated ERK (extracellular-signal-regulated kinase) enzymes rephosphorylate β-arrestin1 at Ser^{412}, providing a mechanism of feedback regulation (see below). Although β-arrestin1 and β-arrestin2 proteins are 78% identical in amino acid sequence, Ser^{412} is unique to β-arrestin1. The phosphorylation of β-arrestin2 has been mapped to a threonine residue (Thr^{383} of rat β-arrestin2 and Thr^{382} of bovine β-arrestin2) and the kinase identified as CKII (casein kinase II) [60,61]. However, the phosphorylation of the bovine protein has been reported not to be linked to any functional consequence other than an interaction with an unknown 100 kDa protein. In the case of rat β-arrestin2, phosphorylation of Thr^{383} regulates the interaction with clathrin. A T383D mutant binds clathrin less efficiently than the wild type. Overexpression of the T383D mutant led to decreased effects on β_2AR internalization as compared with wild-type β-arrestin2, but was not a dominant negative. Furthermore, mutation of Ser^{412} or Thr^{383} in β-arrestin1 or β-arrestin2 respectively, does not alter their properties with respect to receptor interaction and desensitization. Future identification of the phosphatases that are involved may provide important clues to the exact molecular mechanisms of β-arrestin-mediated, clathrin-dependent receptor internalization.

The involvement of individual β-arrestin isoforms in 7MSR internalization has recently been dissected by utilizing mouse embryonic fibroblast cell lines prepared from either single or double β-arrestin knockout mouse embryos [37]. β_2ARs do not internalize in the double knockout cells, whereas 18% of $AT_{1A}R$ sequestration remains in the absence of both β-arrestins. On the other hand, either β-arrestin isoform can support $AT_{1A}R$ sequestration fully, whereas β-arrestin2 is approx. 100-fold more potent than β-arrestin1 in promoting β_2AR sequestration. Some receptors, such as PAR1, show normal sequestration in the double knockout mouse embryonic fibroblast cell lines, and do not appear to depend on β-arrestin for internalization [38].

In general, most 7MSRs bind β-arrestin and are internalized via β-arrestin-dependent mechanisms. Recently developed

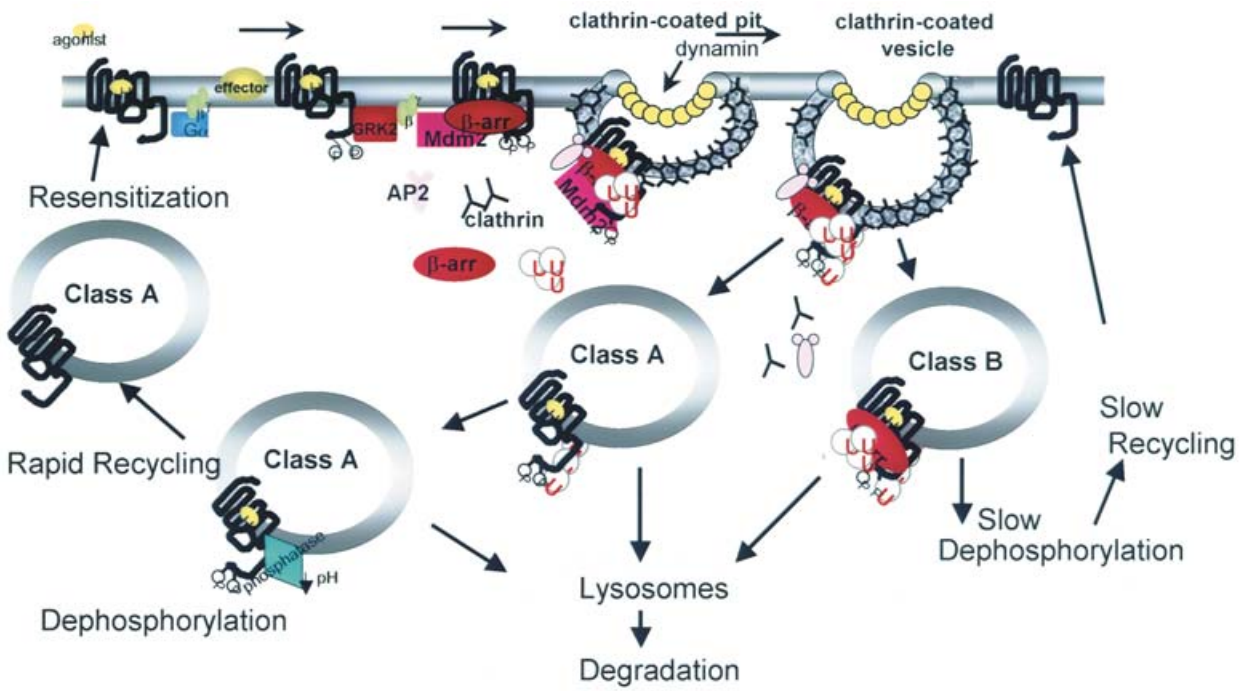

Figure 4 Intracellular trafficking of 7MSRs and β-arrestins

Agonist-dependent phosphorylation of a 7MSR leads to recruitment of β-arrestin (β-arr), whereupon β-arrestin and receptor are both ubiquitinated. The receptor–β-arrestin complex is targeted to clathrin-coated pits. In the case of class A receptors, β-arrestin becomes deubiquitinated, leading to its dissociation from the receptor. The ubiquitinated receptor traffics into late endosomes and lysosomes to be degraded. In the case of class B receptors, β-arrestin remains ubiquitinated and co-localizes with the receptor on endosomes. U, ubiquitin.

GFP-tagged β-arrestin molecules have greatly aided in visualizing the localization of β-arrestins upon agonist stimulation. GFP–β-arrestin1 or GFP–β-arrestin2 is uniformly distributed in the cytosol in the unstimulated condition. Upon stimulation of a 7MSR, GFP–β-arrestins translocate rapidly to the plasma membrane [12,28]. However, two distinct patterns of β-arrestin trafficking within the cell have been delineated, leading to the classification of GPCRs into two classes. Class A receptors (including β_2AR, α_{1b}-adrenergic receptor, μ-opioid receptor, endothelin ET_A receptor and dopamine D_{1A} receptor) show preferential binding to β-arrestin2 compared with β-arrestin1, and no interaction with visual arrestin. Stimulation of these receptors leads to rapid translocation of GFP–β-arrestin to the plasma membrane. β-Arrestin binding to these receptors appears to be transient, since the β-arrestins dissociate from the receptor shortly after movement of the receptor into clathrin-coated vesicles. Hence receptor and β-arrestin complexes do not co-localize in endosomes. Class B receptors (including V_2R, AT_{1A}R, thyrotropin-releasing hormone receptor, neurotensin 1 receptor and neurokinin NK1 receptor) can bind to visual arrestin and show equal affinity for β-arrestin1 and β-arrestin2 [62]. With these receptors, the receptor–β-arrestin complex is more stable. Thus the activated receptor and β-arrestin traffic together and co-localize in endosomes for extended periods (Figure 4). In addition, class A receptors resensitize and recycle more rapidly than class B receptors. Information contained in the C-terminal tail region of a receptor is sufficient to determine its classification. For example, replacing the C-terminal tail of β_2AR (class A) with that of V_2R (class B) or vice versa converts the resulting β_2AR/V_2R or V_2R/β_2AR chimaeras into the other class [31]. Oakley et al. [63] reported the presence of a set of triplet serine residues in the cytoplasmic tails of class B receptors that is responsible for the co-localization of β-arrestin and receptor in endosomes.

Agonist-promoted β-arrestin ubiquitination and receptor endocytosis

Ub (ubiquitin) is a small, ubiquitous, highly conserved protein of 76 residues. Post-translational attachment of Ub, known as ubiquitination, is a highly regulated process wherein the C-terminal glycine residue of Ub becomes covalently attached to the ε-amino group of a lysine residue in a substrate. The process requires three distinct enzyme activities: E1-Ub activating enzyme, E2-Ub carrier enzyme and E3-Ub ligating enzyme [64,65]. A single Ub attachment results in monoubiquitination. Following this, either additional Ub moieties can be attached singly to several lysines (multiubiquitination) or a chain of Ub residues can form on the lysines of the preceding Ub (polyubiquitination). The process of ubiquitination was described decades ago, and the first function to be identified was the targeting of ubiquitinated proteins to a multisubunit megadalton complex called the 26 S proteasome, which functions as a degradation compartment [66,67]. Ubiquitin itself is not degraded, and is cleaved from the substrates by deubiquitinating enzymes.

In recent years, additional roles of ubiquitination and of the proteasomal machinery have been discovered. For example, the precursor of nuclear factor-κB becomes ubiquitinated and targeted to the proteasome, but protein degradation is confined only to the C-terminal region. The N-terminal region survives as the mature p50 form [68,69]. Thus ubiquitination serves to regulate the processing of the precursor to the active form. Ubiquitination also serves as a signal for the sorting of endocytic

cargo into appropriate vesicles that invaginate into multivesicular bodies which ultimately target the cargo to lysosomes (vacuole in case of yeast) for degradation [70].

Recent studies have revealed that both β-arrestin2 and the β_2AR are ubiquitinated in an agonist-dependent manner [71]. Detection of β-arrestin, but not of receptor, ubiquitination requires the inhibition of deubiquitinating enzymes. Interestingly, the oncoprotein Mdm2, a negative regulator of p53, interacts with β-arrestin, as shown by yeast two-hybrid assays, *in vitro* glutathione S-transferase pull-downs and cellular co-immunoprecipitation. Constitutive interaction occurs at both endogenous and overexpressed levels of the two proteins. Mdm2 contains a RING (really interesting new gene) domain at its C-terminus and is an E3-Ub ligase [71a,71b]. Mdm2-catalysed ubiquitination of β-arrestin is essential for receptor internalization. β_2AR ubiquitination is not required for its internalization, since a mutant receptor that has no ubiquitinatable lysines is internalized normally [71]. Receptor ubiquitination, which interestingly requires β-arrestin2, perhaps as an adapter to bring E3-ligases to proximity, is required for the proper sorting and degradation of the internalized receptor in lysosomes. Similar ubiquitination-dependent sorting to lysosomes has been reported in the case of the HIV co-receptor CXCR4 (CXC chemokine receptor 4). Mutation of lysines in the tail region eliminated both ubiquitination and degradation, but not internalization, of the CXCR4 receptor [72]. Currently, the specific ligase acting on the β_2AR or other mammalian 7MSRs is unknown. In the case of the β_2AR, although Mdm2 is capable of catalysing its ubiquitination, receptor ubiquitination and degradation still occur in Mdm2 null cells. This indicates that there must be another, as yet unidentified, ligase present in the receptor–β-arrestin complex [73].

In the case of yeast 7MSR Ste2 (α factor receptor), internalization and subsequent degradation in the vacuole require phosphorylation and monoubiquitination of the receptor [74,75]. Furthermore, an E3-Ub ligase, Rsp5p, is required at multiple steps of Ste2 endocytosis. Thus a Ste2–Ub chimaera and Ste2 substituted with Ub-independent internalization motifs are unable to internalize in Rsp-deficient yeast cells [76,77]. In the case of Ste3 (a factor receptor) in yeast, although monoubiquitination suffices, polyubiquitination enhances the rate of endocytosis of the receptor [78]. Arrestin and GRK homologues, however, are absent from yeast.

β-Arrestin deubiquitination and intracellular trafficking

A striking feature of β-arrestin ubiquitination is the differing kinetics of deubiquitination with respect to the two classes of receptors described above. Stimulation of class A receptors such as β_2AR, and $V_2R\beta_2CT$ (V_2R with the C-terminal residues of β_2AR) [31] leads to transient β-arrestin ubiquitination, whereas when a class B receptor such as V_2R or β_2ARV_2CT (β_2AR with the C-terminal residues of V_2R) [31] is stimulated, β-arrestin remains stably ubiquitinated [27]. The receptor–β-arrestin interaction during receptor trafficking also correlates with the ubiquitination status of the β-arrestin moiety after agonist stimulation. Thus transient ubiquitination of β-arrestin upon stimulation by isoprenaline leads to a transient receptor–β-arrestin interaction at the plasma membrane, whereas stable ubiquitination of β-arrestin following V_2R stimulation leads to co-localization of receptor–β-arrestin complexes on endosomes. Thus the time course of β-arrestin ubiquitination and deubiquitination parallels its association with and dissociation from the receptor (Figure 4). Furthermore, class A receptors can be transformed into the class B type by a β-arrestin2–Ub chimaera. This chimaeric protein is neither efficiently deubiquitinated nor dissociated from the internalizing β_2AR, unlike wild-type β-arrestin2, which is deubiquitinated and dissociates from the β_2AR shortly after movement into clathrin-coated pits. The association of β-arrestin–Ub and β_2AR in endocytic vesicles also leads to enhanced receptor internalization and degradation. Thus, apart from their critical role in receptor endocytosis, β-arrestin ubiquitination and deubiquitination kinetics induced by distinct 7MSRs also determine the characteristic patterns of intracellular trafficking.

The fate of ubiquitinated β-arrestin appears to be different after chronic insulin treatment of Rat-1 fibroblast cells overexpressing the insulin receptor. In this case, after a 12 h treatment with insulin (100 ng/ml), ubiquitinated β-arrestin1 was degraded by the proteasome, leading to a decrease in the MAPK (mitogen-activated protein kinase) signal mediated by IGF-1 (insulin-like growth factor 1), lysophosphatidic acid and isoprenaline [79]. Additionally, in 3T3-L1 adipocytes, similar insulin treatment and a decrease in β-arrestin levels leads to super-sensitization of $G\alpha_s$-associated signalling and inhibition of $G\alpha_i$-associated signalling of the β_2AR [80].

Endocytic roles of β-arrestin beyond traditional 7MSRs

The endocytic function of β-arrestin has recently been extended to other families of receptors, such as the IGF-1 receptor and the Frizzled family of 7MSRs. β-Arrestin1 binds to the agonist-occupied IGF-1 receptor and promotes receptor internalization via a clathrin-coated pathway [81]. β-Arrestin2 also regulates the endocytosis of the 7MSR Fz4 (Frizzled 4). The Frizzled family of cell surface receptors are stimulated by glycoprotein molecules of the Wnt family (Wingless or Wg in the fruit fly *Drosophila*), leading to stabilization of β-catenin in the cytosol and increased activity of LEF (lymphoid enhancer factor)/Tcf (T-cell factor) transcription factors. The interaction of Wnt and Fz4 also results in the recruitment of a cytoplasmic protein, Dvl (Dishevelled). Interestingly, β-arrestins can interact with Dvl. *In vitro* binding of the two proteins, β-arrestin1 and Dvl, is enhanced severalfold when Dvl is phosphorylated [82].

In general, 7MSRs recruit β-arrestin after agonist stimulation, whereupon β-arrestin serves as an adapter to bring various other molecules into complex with the receptor. However, in the case of Fz4, Dvl recruits β-arrestin upon Wnt5A treatment [83]. Internalization of Fz4–GFP occurs via clathrin-coated vesicles and is observed upon concurrent stimulation with both Wnt5a and phorbol esters (that activate PKC). Silencing the β-arrestin2 gene with siRNA ablates Fz4–GFP internalization, suggesting that stimulation of Fz4 receptors leads to PKC-mediated phosphorylation of Dvl, resulting in β-arrestin recruitment to the receptor–Dvl complex and clathrin-dependent internalization of the Fz4 receptor.

EXPANDED ROLES OF β-ARRESTINS: SIGNALLING THROUGH KINASES

Although β-arrestin proteins were discovered in the context of dampening of receptor signal transduction, recently it has been appreciated that they also initiate signals from the very receptors that they 'desensitize'. Thus an ever-increasing list of kinases and other regulatory proteins that bind specifically to either one or both β-arrestin isoforms has been discovered. Hence β-arrestins act as adapters for Src-family tyrosine kinases, and as receptor-regulated scaffolds for several ERK, JNK (c-Jun N-terminal kinase) and p38 MAPK modules (Figure 5).

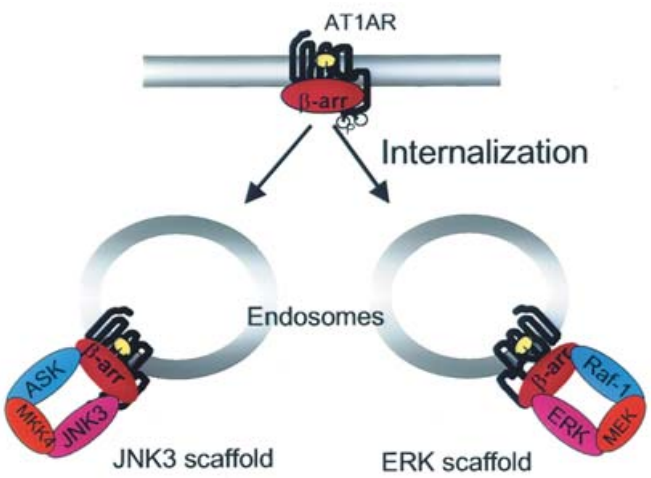

Figure 5 β-Arrestin, a 7MSR-regulated scaffold

Stimulation of class B receptors leads to GRK-mediated phosphorylation and recruitment of β-arrestin (β-arr). By virtue of its scaffolding functions, β-arrestin brings members of MAPK cascades to form complexes with the receptor, leading to 7MSR-dependent MAPK signalling.

β-Arrestin and non-receptor tyrosine kinases

Many 7MSRs mediate the Ras-dependent activation of mitogenic signalling pathways, requiring the recruitment and activation of Src-family non-receptor tyrosine kinases. Upon recruitment to the plasma membrane, c-Src tyrosine kinase phosphorylates the adapter protein Shc, leading to the recruitment of the Ras exchange factor Sos and its adapter Grb2, and subsequent activation of Ras, Raf-1, MEK1 (MAPK/ERK kinase 1) and finally ERK1/2 [84].

Initial evidence that β-arrestin acts as a signalling adapter came from studies that demonstrated isoprenaline-stimulated, β-arrestin-dependent, c-Src recruitment to the membrane [24]. Two domains on c-Src mediate β-arrestin binding. The SH3 (Src homology 3) domain interacts with proline-rich regions of β-arrestin, and the kinase SH1 domain binds to the N-terminal region of β-arrestin. An inactive c-Src mutant (K298M, with a point mutation in the ATP binding site) consisting of the SH1 domain alone (residues 250–536) binds β-arrestin, but is unable to function at the membrane upon recruitment. This mutant, called SH1KD, acts as a dominant-negative with respect to processes requiring both Src activity and β-arrestin recruitment [85]. Isoprenaline stimulation of the β_2AR leads to the c-Src-mediated tyrosine phosphorylation of dynamin at the membrane at two residues, Tyr^{231} and Tyr^{597}, which is required for β_2AR endocytosis and isoprenaline-stimulated ERK-mediated phosphorylation [86]. The c-Src mutant SH1KD blocks both c-Src-mediated tyrosine phosphorylation of dynamin and β_2AR sequestration in HEK 293 and COS-7 cells [85]

Agonist stimulation of the neurokinin 1 receptor in Kirsten sarcoma virus-transformed rat kidney epithelial cells leads to robust ERK activation at the membrane upon the formation of a multiprotein complex consisting of the receptor, β-arrestin, c-Src and ERK1/2 [87]. Phosphorylation of ERK is dependent on recruitment of β-arrestin to the receptor, since a truncated neurokinin 1 receptor that fails to recruit β-arrestin does not activate ERK.

Stimulation of the 7MSR endothelin ET_A receptor with endothelin 1 results in the formation of a molecular complex composed of the receptor, β-arrestin1, $G\alpha_{q/11}$ and the Src tyrosine kinase Yes, leading to the translocation of GLUT4 and stimulation of glucose transport [88]. Olefsky and colleagues [88] have demonstrated that, in 3T3-L1 adipocytes, endothelin 1-stimulated GLUT4 translocation is sensitive to microinjection of antibodies to either β-arrestin1 or the Src kinase Yes. Furthermore, the dominant-negative SH1KD c-Src mutant blocks endothelin 1-stimulated GLUT4 translocation, confirming the requirement for recruitment of β-arrestin–Src in this process [88].

β-Arrestins also play a critical role in the regulation of granule release from interleukin-8-stimulated leucocytes [89]. Barlic and co-workers [89] reported that β-arrestins interact with Hck and c-Fgr (Src-family tyrosine kinases) upon interleukin-8 stimulation of human neutrophils or CXCR1-expressing RBL cells. The β-arrestin–kinase complex translocates to the granular compartment and initiates steps leading to exocytosis and release of granules. The entire process requires recruitment of β-arrestin to the C-terminal tail of the receptor CXCR1, which in turn is dependent on the phosphorylation of serine/threonine clusters by GRKs and PKC. Mutation of all of the distal serine and threonine residues in the receptor resulted in no granular release upon interleukin-8 treatment, consistent with the hypothesis that β-arrestin recruitment to the phosphorylated receptor indeed initiates the chemokine responses.

β-Arrestin and MAPK activation

The family of MAPKs includes ERK1 ($p44^{MAPK}$), ERK2 ($p42^{MAPK}$), ERK5 (big MAPK or BMK), JNK1–JNK3 [also called SAPKs (stress-activated protein kinases)] and p38 MAPKs (α, β, γ and δ isoforms). MAPKs phosphorylate a variety of protein substrates and regulate numerous cellular processes, including cell proliferation, gene expression and apoptosis. MAPKs are serine/threonine kinases that are phosphorylated by upstream MAPKKs (MAPK kinases), which in turn are activated by the MAPKKKs (MAPKK kinases). Eukaryotic cells have numerous such serially activated protein kinases forming MAPK cascades. In several instances, the same protein kinase can be part of more than one pathway, which can potentially lead to unwanted cross-talk between different pathways. In order to maintain signal specificity, members of a particular cascade are often tethered together by regulatory scaffolding proteins. The first scaffold to be described was yeast Ste5p, which binds members of a MAPK cascade, namely Ste11p (MAPKKK), Ste7p (MAPKK) and Fus3p or Kss1p (MAPK) [90]. Pheromone (α factor) activation of the yeast GPCR triggers activation of G-proteins and translocation of Ste5p to the $G\beta\gamma$ locale at the plasma membrane. This results in a stable Ste5p signalling scaffold, leading ultimately to the activation of Fus3p/Kss1p. A mammalian homologue of Ste5p has not been defined. However, several mammalian scaffold proteins have been described that function in a manner that is seemingly analogous to Ste5p, e.g. the JIP (JNK-interacting protein) family of proteins [JIP1, JIP2 and JIP3/JSAP1 (switch-activating protein 1)] that act as scaffolds for regulation of the JNK/SAPK pathway [91,92].

Recent data demonstrate the scaffolding functions of β-arrestin in the JNK3 and ERK1/2 MAPK modules. JNK3, identified as a β-arrestin binding partner in a yeast two-hybrid screen, interacts with β-arrestin at endogenous levels of protein expression [93]. β-Arrestin overexpression in HEK 293 cells causes retention of the JNK3 protein in the cytosol. Furthermore, ASK1 (apoptosis signal-regulating kinase 1), one of the MAPKKKs that can activate JNKs, as well as MKK4, a MAPKK, forms a protein complex with β-arrestin and JNK3. Stimulation of the $AT_{1A}R$ in COS-7 cells results in JNK3 activation in a β-arrestin-dependent manner, and receptor, β-arrestin2 and phospho-JNK3 co-localize

on endocytic vesicles. Thus β-arrestin not only brings together the appropriate kinases of a specific module, but also scaffolds these kinases under the guidance of an activated 7MSR.

The generally defined substrates of JNK enzymes reside in the nucleus. What then is the purpose of β-arrestin's retention of the kinase in the cytosol? It is possible that JNK3 has as yet unidentified specific substrates in the cytosol. In addition, the JNK proteins become rapidly dephosphorylated on entering the nucleus. Retention in the cytosol by a β-arrestin scaffold might prolong the lifespan of the active form of the kinase. Future identification of specific β-arrestin-dependent JNK3 substrates or processes in the cytosol will help to improve our understanding of the β-arrestin/JNK3 MAPK module.

β-Arrestins also act as 7MSR-regulated scaffolds for ERK1/2, thus bringing together Raf (MAPKKK), MEK1 (MAPKK) and ERK (MAPK). Stimulation of $G\alpha_q$-coupled PAR2 leads to the formation of a multiprotein signalling complex consisting of β-arrestin, Raf-1 and ERK, cytosolic retention of phosphorylated ERK and co-localization of receptor–β-arrestin–ERK complexes on endocytic vesicles [94]. A mutant PAR2 receptor in which the potential phosphorylation sites in the cytoplasmic tail are changed to alanines (PAR2δST363/6A) is defective in both internalization and desensitization, most probably due to a retarded interaction with β-arrestin. This mutant does activate ERK upon stimulation through a Ras-independent pathway, leading to nuclear translocation of phosphorylated ERK and mitogenic signalling. In this case, the ability of the activated PAR2 to complex with β-arrestin appears to be the determinant of whether ERK activation occurs through Ras-dependent (wild-type receptor) or Ras-independent (PAR2δST363/6A) mechanisms.

Another $G\alpha_q$-coupled receptor that can engage β-arrestin/Raf/MEK/ERK scaffolds in the cytoplasm is the $AT_{1A}R$ [95]. Stimulation of the $AT_{1A}R$ in COS-7 or HEK 293 cells results in co-localization of receptor, β-arrestin and ERK2–RFP (red fluorescent protein) in endosomal vesicles, as demonstrated by confocal microscopy. Co-immunopreciptation assays demonstrate formation of a complex comprising β-arrestin2, c-Raf-1, MEK1 and ERK2–GFP. Either stimulation of $AT_{1A}R$ or c-Raf-1 overexpression can lead to enhancement of the formation of this multiprotein complex. Furthermore, overexpression of β-arrestin1 or β-arrestin2 in COS-7 cells causes a marked decrease in angiotensin-stimulated phosphatidylinositol hydrolysis, and a prominent increase in ERK phosphorylation. This activated pool of ERK is complexed with β-arrestin and retained in the cytosol, which results in a significant decrease in the ERK1/2-mediated, Elk1-driven transcription of a luciferase reporter [96]. These effects of β-arrestin on ERK phosphorylation can also be observed following overexpression of the upstream MAPKKK c-Raf-1, even in the absence of $AT_{1A}R$ expression.

The retention of activated ERK complexed with β-arrestin on endosomes is also observed with other class B receptors. Thus, in addition to the $AT_{1A}R$, agonist stimulation of the V_2R or of the chimaeric β_2AR with a V_2R C-terminal tail (β_2ARV2CT), which can form stable receptor–β-arrestin complexes, efficiently activates a β-arrestin-bound pool of ERK2 [97]. This is in contrast with class A receptors, such as the α_{1b}-adrenergic receptor, β_2AR and the chimaeric V_2R with the β_2AR C-terminal tail ($V_2R\beta2CT$), that bind β-arrestin transiently [97]. Stimulation of receptors that stably bind β-arrestin (class B) leads to cytosolic retention of activated ERK and to a decrease in the nuclear translocation of phospho-ERK1/2. On the other hand, receptors that bind β-arrestin only at the plasma membrane and do not co-localize with β-arrestin on endosomes can lead to greater ERK-mediated nuclear responses of Elk1-driven transcription reporters, as well as to an increase in the mitogenic response. Such a transient β-arrestin-binder (e.g. β_2AR) can be transformed to stably bind β-arrestin merely by replacing the tail residues with those of a stable β-arrestin binder such as the V_2R. Tohgo et al. [97] demonstrated that this interconversion of receptor types also occurs in terms of β-arrestin-mediated ERK activation. Thus the distinct effects of the spatial and temporal regulation of activated pools of ERK are a net result of the interaction between β-arrestin and the cytoplasmic tail of a particular receptor. The physiological role of phospho-ERK in the cytosol is not fully understood. In addition to phosphorylating transcription factors in the nucleus, ERK1/2 phosphorylate a growing list of cytoskeletal, plasma membrane and cytosolic proteins, such as β-arrestin1 [98], GRK2 [99], Gα-interacting protein [100], $p90^{RSK}$ (where RSK is p90 ribosomal S6 kinase) [101] and many others.

Although a large number of pathways for ERK activation appear to exist in mammalian cells, the current experimental data indicate the presence of a specific 7MSR-stimulated pathway that requires the β-arrestin proteins. However, the extent of interdependence or cross-talk between the β-arrestin-dependent and G-protein-dependent pathways is unclear at present. β-Arrestin binding to 7MSRs generally requires GRK-mediated phosphorylation of the receptor. The receptor kinases GRK2 and GRK3 are dependent on the $G\beta\gamma$ subunits for their membrane attachment and activation. Thus, at least in the case of 7MSRs that are specifically phosphorylated by GRK2 and GRK3 enzymes, β-arrestin binding to the receptor and subsequent scaffolding may still require a G-protein component.

β-Arrestin also serves to mediate the activation of p38 MAPK via 7MSR stimulation. β-Arrestin plays a role in augmenting the p38 MAPK signalling of the chemokine receptor CXCR4. β-Arrestin-dependent p38 activation, but not ERK activation, is crucial for SDF (stromal cell-derived factor)-induced chemotaxis in transfected HEK 293 cells [102]. β-Arrestin2-dependent p38 MAPK signalling has also been observed in the case of the human cytomegalus virus-encoded viral GPCR US28. US28 displays constitutive activity as well as β-arrestin recruitment in cells, even in the absence of agonist. A truncated US28 is impaired in both β-arrestin binding and p38 MAPK signalling [103].

ROLE OF β-ARRESTIN IN CHEMOTAXIS

Chemotaxis is defined as the directed migration of cells towards agonists. This directional sensing of a stimulus, followed by cell migration towards the stimulus, forms the key determinant of immune reactions, wound healing, embryogenesis, angiogenesis and neuronal patterning [104]. In lymphocytes, 7MSRs that couple to $G\alpha_i$ proteins regulate the initiation of chemotactic responses. Chemotaxis requires $G\beta\gamma$ but not $G\alpha$ subunits. Other proteins that are crucial for directed migration are Rho GTPases, phosphoinositide 3-kinases and ERK1/2. The classical role of β-arrestins in this G-protein-dependent pathway would be to desensitize or to stop the G-protein-mediated chemotaxis. However, recent work by Fong et al. [105] showed that this is not the case. In their studies, as expected, splenocytes from βarrestin2- and GRK6-deficient animals showed increased membrane GTPase activity in response to CXCL12 stimulation, suggesting impaired desensitization [105]. Quite unexpectedly, however, trans-well and trans-endothelial migration assays performed with lymphocytes from either β-arrestin2 or GRK6 knockout mice showed *impaired* chemotaxis with respect to the agonist CXCL12 (also known SDF-1). Thus the directed movement of lymphocytes towards chemokines is regulated specifically by GRK6-mediated phosphorylation and β-arrestin2 binding to the 7MSR CXCR4 (a chemokine receptor; also a HIV co-receptor).

Very recently, DeFea's group [106] reported that activation of PAR2 (a 7MSR that is abundant in neutrophils, macrophages and tumour cells) can lead to actin/cytoskeletal reorganization, pseudopod extension and chemotaxis in a β-arrestin- and ERK-dependent manner. Using subcellular fractionation, confocal microscopy and isolation of pseudopodial proteins, they demonstrated that the cellular extensions towards agonist are enriched in β-arrestin-scaffolded phospho-ERK. Similar chemotactic responses were not detected by stimulating 7MSRs that activate ERK independent of β-arrestin, such as PAR1. In the case of PAR2, these novel findings imply a role for β-arrestin-sequestered cytoplasmic ERK activity in cell motility.

ROLE OF β-ARRESTINS IN APOPTOTIC PATHWAYS

The β-arrestin2 homologue in the fly, arrestin2, is linked to apoptotic pathways during retinal development [107–109]. The *Drosophila* compound eye is a model system for studying photoreception via the GPCR rhodopsin. Rhodopsin activation leads to activation of $G\alpha_q$ and of the phospholipase C norpA. Receptor phosphorylation by rhodopsin kinase leads to arrestin2 binding and desensitization. The dephosphorylation of rhodopsin by the phosphatase rdgC and phosphorylation of arrestin2 by calcium/calmodulin-dependent kinase II leads to the disassembly of receptor–arrestin2 complexes. Light-dependent retinal degeneration occurs in norpA mutants as well as in rdgC mutants, which are defective in dephosphorylation of meta-rhodopsin. This apoptotic pathway is dependent on the formation of stable complexes of rhodopsin and arrestin2, as well as on the endocytosis of such complexes via a clathrin- and dynamin-dependent pathway. In both norpA and rdgC mutant backgrounds, knocking out either rhodopsin or arrestin prevents retinal degeneration. Light-dependent retinal degeneration akin to necrosis also occurs in arrestin2 mutants, which is suppressed by the elimination of $G\alpha_q$ protein. Thus two distinct retinal degeneration pathways are defined: a G-protein-dependent pathway that is necrotic but not apoptotic, and an arrestin2-dependent pathway that is apoptotic [110]. Recently, Acharya et al. [111] have demonstrated a role for ceramide, functioning as an agent in regulating both the G-protein-dependent necrotic pathway and the arrestin2-dependent apoptotic pathway. Expression of ceramidase, an enzyme that hydrolyses ceramide to sphingosine, reverses the photoreceptor cell death in both arrestin2 and norpA mutants. Targeted disruption of the *Lace* gene, encoding the LCB2 subunit of serine palmitoyl-CoA transferase (which catalyses the initial step of ceramide biosynthesis), also suppressed the photodegenerative effects of arrestin2 and norpA mutations. These studies implicate the endocytic machinery as playing a major role in the degenerative processes. Thus the prolonged retention of arrestin2 complexed with rhodopsin in multivesicular bodies somehow initiates steps leading to increased ceramide levels, by either up-regulation of its synthesis or down-regulation of its destruction, and eventual programmed cell death. These provocative findings should lead to future research geared towards greater understanding of several forms of inheritable retinal degenerative diseases that result from mutations in rhodopsin, arrestin and other components of the phototransduction cascade.

Wang et al. [112] reported an interesting role for β-arrestin in p53-mediated apoptosis by overexpressing different proteins in p53-null human osteosarcoma cells (Saos2 cells). Exogenous expression of p53 in Saos2 cells leads to approx. 60 % apoptosis, which is decreased by co-expression of Mdm2. Interestingly, overexpression of β-arrestin2 decreased Mdm2-mediated p53 degradation and enhanced p53-regulated cellular apoptosis. In the same cells, depletion of endogenous β-arrestin expression by RNAi (RNA interference) methods attenuated p53-mediated apoptosis. These studies suggest that levels of expression of β-arrestin in cells can regulate p53-mediated apoptotic pathways.

NUCLEOCYTOPLASMIC SHUTTLING OF β-ARRESTINS

Earlier studies with GFP and YFP (yellow fluorescent protein)-tagged β-arrestin1 and β-arrestin2 revealed an important difference in the subcellular distribution of the two isoforms [62]. Thus β-arrestin1 is located in both the cytosol and nucleus, whereas β-arrestin2 is excluded from the nucleus and is purely cytosolic. Visual arrestin has a similar distribution as β-arrestin1. Recent reports from Scott et al. [113] and Wang et al. [114] have shed new light on β-arrestin nucleocytoplasmic shuttling. β-Arrestin2 is constitutively excluded from the nucleus due to the presence of a leucine-rich nuclear export signal at the C-terminus. The shuttling of β-arrestin2 occurs via a leptomycin B-sensitive pathway. Although the exact role(s) of β-arrestin2 in the nucleus is currently unknown, it is possible that the scaffolding and signalling functions of β-arrestin2 might require its transit through the nucleus, analogous to Ste5p in yeast [115].

CONCLUSIONS

The diverse roles of β-arrestins summarized above have been discovered in the past decade. However, much remains to be discovered. Initially thought to be almost interchangeable, differences between β-arrestin1 and β-arrestin2 in affinity, both for different receptors and for some effectors (e.g. JNK3), have begun to emerge. Delineation of their unique biological properties is an important goal of future studies. Whereas emphasis in the past has been on studying roles of β-arrestin in desensitization, now the spotlight has shifted towards their multifaceted adapter and scaffolding functions in receptor endocytosis and in signalling pathways. It would appear that current information represents only the very tip of the iceberg with respect to these mechanisms. Finally, although β-arrestins are generally regarded as exclusively regulating 7MSRs, recent findings suggest that their full range of receptor targets may indeed be much broader.

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