

β -Arrestins and Cell Signaling

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Annu. Rev. Physiol. 2007. 69:483–510

The *Annual Review of Physiology* is online at
<http://physiol.annualreviews.org>

This article's doi:
10.1146/annurev.physiol.69.022405.154749

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0066-4278/07/0315-0483\$20.00

Key Words

seven-transmembrane receptor, G protein-coupled receptor
kinase, scaffold, phosphorylation, ERK, MAPK

Abstract

Upon their discovery, β -arrestins 1 and 2 were named for their capacity to sterically hinder the G protein coupling of agonist-activated seven-transmembrane receptors, ultimately resulting in receptor desensitization. Surprisingly, recent evidence shows that β -arrestins can also function to activate signaling cascades independently of G protein activation. By serving as multiprotein scaffolds, the β -arrestins bring elements of specific signaling pathways into close proximity. β -Arrestin regulation has been demonstrated for an ever-increasing number of signaling molecules, including the mitogen-activated protein kinases ERK, JNK, and p38 as well as Akt, PI3 kinase, and RhoA. In addition, investigators are discovering new roles for β -arrestins in nuclear functions. Here, we review the signaling capacities of these versatile adapter molecules and discuss the possible implications for cellular processes such as chemotaxis and apoptosis.

7TMR: seven-transmembrane receptor

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

β 2AR: β -2 adrenergic receptor

Adapter: a protein that enhances cellular responses by recruiting key proteins into a complex

AT1_AR: angiotensin II receptor type 1A

β -ARRESTINS: DISCOVERY, BIOLOGY, AND CLASSICAL FUNCTIONS

The classic paradigm of signal transduction in response to stimulation of seven-transmembrane receptors (7TMRs), also known as G protein-coupled receptors (GPCRs), involves an agonist-induced conformational change that allows the receptor to interact with and dissociate the G α from the G $\beta\gamma$ subunits of heterotrimeric G proteins (for a historical review, see Reference 1). Distinct subtypes of G α proteins, such as G α_s , G α_q , and G α_i , signal through discrete pathways via second messenger molecules such as cyclic AMP, inositol triphosphate, diacylglycerol, and calcium. Members of a protein family known as the G protein-coupled receptor kinases (GRKs) initiate the termination of this signaling response (for reviews see References 2–4). GRKs rapidly phosphorylate the receptor, typically on its cytoplasmic tail. β -Arrestins then bind the phosphorylated receptor, which blocks further G protein-initiated signaling through a steric mechanism.

Discovery of β -Arrestins

The discovery of β -arrestins in the late 1980s resulted from the observation that increasingly pure preparations of GRK2 (then referred to as β adrenergic receptor kinase) progressively lost the ability to desensitize G protein activation in a reconstituted β -2 adrenergic receptor (β 2AR) system (5). The loss of desensitization could be rescued by addition of high molar excesses of visual arrestin (S-antigen or 48-kDa protein) (6), which researchers had recently discovered to function together with rhodopsin kinase to desensitize rhodopsin signaling. This implied that a homologous protein could exist in nonretinal tissues (5). Molecular cloning confirmed this and revealed two isoforms of the hypothesized protein, termed β -arrestins 1 and 2 (or arrestins 2 and 3) because of their homology

with arrestin and their functional regulation of the β 2AR (7, 8).

β -Arrestins as Adapters for Internalization

β -Arrestins are expressed ubiquitously in all cells and tissues and function in the desensitization of most 7TMRs except rhodopsin. Although their role in the termination of signaling led to their discovery, later research appreciated that β -arrestins serve a second function in receptor internalization [for a review, see Benovic et al. (8a) in this volume]. By acting as adapters for β (2) adaptin, better known as AP2, and clathrin (9–11), β -arrestins bring activated receptors to clathrin-coated pits for endocytosis, a process critical for receptor recycling and degradation. β -Arrestins also bind to various other proteins implicated in receptor internalization. For example, β -arrestin2 is constitutively bound to the guanine nucleotide exchange factor ARNO (ARF nucleotide binding site opener) and serves as a switch to regulate the activity of the small G protein ARF6 (ADP-ribosylation factor 6), which is bound to β -arrestin2 only upon receptor stimulation (12, 13). When ARNO activates ARF6, the latter is released by β -arrestin2 and assists in the endocytosis of the receptor.

Isoform Differences Between β -Arrestins 1 and 2

The amino acid sequences of the two β -arrestin isoforms are 78% identical; most of the coding differences appear in the C termini. Knockout studies show that mice lacking either β -arrestin1 or -2 are viable (14, 15), whereas the double-knockout phenotype is embryonic lethal (16; R.J. Lefkowitz & F.T. Lin, unpublished data), implying that each β -arrestin functionally substitutes for the other isoform to some degree. However, the molecular studies reviewed here do not support redundant roles for all β -arrestin-mediated functions. For example, internalization of

Table 1 7TMRs and their β -arrestin-dependent properties^a

Receptor		β -Arrestin recruitment	β -Arrestin-dependent ERK	β -Arrestin isoform regulation of ERK	Cell type	Reference
AT _{1A} R	Wild type	Class B	Yes	Reciprocal*	HEK-293	58
	Δ 324	Class A	Yes	N.D.	HEK-293	Figure 4
β 2AR	Wild type	Class A	Yes	Codependent	HEK-293	61
	GRK ⁻ , PKA ⁻	None	No	N.A.	HEK-293	Figure 4
PAR2	Wild type	Class B	Yes	Codependent	HeLa	118
	C-tail deletion	Class A	No	N.A.	HeLa	
V2R		Class B	Yes	Reciprocal*	HEK-293	60
PTH1R		Class B	Yes	Codependent	HEK-293	62
CCR7		Class B	Yes	N.D.	HEK-293	67
PAR 1		N.D.	Yes	Reciprocal**	HEK-293	114

^aReceptors are designated as recruiting β -arrestins in either a Class A (transient, weak interaction; results in rapid recycling of receptors after internalization) or Class B interaction (strong and long lasting; receptors internalize with β -arrestins, and thus slow recycling occurs). Next, receptors are classified as to whether or not the wild-type or mutant receptor retains the ability to activate ERK via β -arrestins. β -Arrestin isoform specificity for ERK activation is listed in the fourth column: Either both β -arrestin isoforms are required for ERK signaling (termed codependent), or only one particular isoform is required, and the other isoform serves to inhibit ERK signaling (termed reciprocal regulation). One asterisk denotes that β -arrestin2 carries the signal to ERK and β -arrestin1 serves to inhibit. Two asterisks denote that β -arrestin1 functions to signal and β -arrestin2 inhibits. N.D., no data; N.A., not applicable.

some 7TMRs is mediated primarily by one isoform, as is the case for the β 2AR via β -arrestin2, whereas for others, like the angiotensin II type 1A receptor (AT_{1A}R), both β -arrestin isoforms are equally capable (16, 17). Likewise, either β -arrestin isoform is capable of AT_{1A}R second messenger desensitization, and the two can functionally substitute for each other, but for the protease-activated receptor 1 (PAR1), only β -arrestin1 can desensitize phosphoinositide turnover (18).

Two Patterns of β -Arrestin Recruitment

Upon 7TMR stimulation and subsequent β -arrestin recruitment to the cytoplasmic membrane, two patterns emerge. For some receptors, there is transient, low-affinity binding characterized by a rapid concentration of β -arrestin at the activated receptor. β -Arrestin is subsequently released after targeting the receptor to clathrin-coated pits. This pattern, termed Class A, is typified by the β 2AR. Class A receptors typically undergo rapid recycling to the plasma membrane after their internalization. In contrast, Class B receptors, such as

the AT_{1A}R, show a much stronger and more prolonged binding to β -arrestin, such that following recruitment to clathrin-coated pits, the receptor and β -arrestin remain bound together on the surface of endocytic vesicles (17). Because of their prolonged interaction with β -arrestins, Class B receptors recycle to the cell surface much more slowly than Class A receptors. **Table 1** summarizes β -arrestin recruitment patterns for various 7TMRs.

β -Arrestin Regulation by Phosphorylation and Ubiquitination

Mammalian arrestins exist in a constitutively phosphorylated state in the cytosol and are dephosphorylated upon binding activated 7TMRs at the plasma membrane. For β -arrestin1, serine 412 is the site of extracellular signal-regulated kinase (ERK) 1/2-mediated phosphorylation (19). In contrast, for β -arrestin2, which is phosphorylated by casein kinase II, threonine 383 is the primary phosphorylation site, and serine 361 represents a secondary site (20–21). Dephosphorylation of β -arrestins at the plasma membrane is necessary for engaging endocytic partners

ERK: extracellular signal-regulated kinase

Class A interaction: weak and transient interaction of β -arrestins and receptors after agonist binding; receptors undergo rapid recycling back to the membrane after internalization

Class B interaction: strong and long lasting interaction of β -arrestins and receptors after agonist binding; after β -arrestins and receptors internalize together, the receptors undergo slow recycling back to the membrane

Ubiquitination: the covalent attachment of ubiquitin, a 76-amino-acid-residue-containing protein, to lysine residues in the substrate protein

such as clathrin but does not seem to affect receptor binding or desensitization (22). In fact, S412D β -arrestin1, which mimics the phosphorylated form of β -arrestin1, acts as a dominant-negative mutant with respect to receptor internalization (22). Presumably, β -arrestins are rephosphorylated by kinases associated with the internalizing complexes.

β -Arrestins also undergo ubiquitination upon activation of several 7TMRs (23–26). Ubiquitination results from a three-step enzymatic process in which a small protein, ubiquitin, is covalently appended to lysine residues in the substrate proteins (27, 28). Ubiquitination is the result of the sequential action of three enzymes. First, the formation of a high-energy thioester bond by a ubiquitin-activating enzyme, E1, activates the COOH-terminal glycine residue of ubiquitin. Activated ubiquitin is then transferred to an active-site cysteine residue in an E2 (ubiquitin-carrying enzyme). The final step is catalyzed by E3, a ubiquitin protein ligase, which links the COOH terminus of ubiquitin to the ϵ -amino group of a lysine residue of the substrate protein.

Although originally discovered to function as a protein tag for destruction by the cellular proteasomal machinery, ubiquitin modification is now appreciated for mediating novel outcomes including protein trafficking and signal transduction (29–33). Agonist stimulation of the β 2AR leads to β -arrestin ubiquitination, which is mediated by Mdm2, a RING domain containing E3 ubiquitin ligase. β -Arrestin ubiquitination is required for rapid receptor internalization (23). Remarkably, the nature and stability of receptor-arrestin complexes formed upon agonist stimulation reflect the status and longevity of β -arrestin ubiquitination. Stimulation of Class A receptors such as the β 2AR leads to transient ubiquitination of β -arrestin (24, 26). In contrast, stimulation of Class B 7TMRs, which recruit β -arrestin to the plasma membrane and subsequently internalize as receptor-arrestin complexes into endosomes, results in sustained β -arrestin ubiqui-

tion (24, 26). The phosphorylation status of the receptor carboxyl tail is one determinant of tight (Class B) versus loose (Class A) binding of β -arrestin thus governing the characteristic endosomal and plasma membrane recruitment patterns. However, these patterns of β -arrestin trafficking are also dictated by the attachment of ubiquitin moieties to specific lysines (e.g., lysines 11 and 12 for the class B AT1_AR) within β -arrestin2 (25). Furthermore, when ubiquitin is translationally fused to the C terminus of β -arrestin, endosomal localization occurs even with Class A receptors (24, 25). Thus, β -arrestin ubiquitination status likely plays a key role in determining the stability of the interactions of β -arrestin with 7TMRs and perhaps other elements of the endocytic machinery. These downstream interactions directed by the ubiquitination status of β -arrestin may in turn dictate the trafficking itinerary of endocytosed receptor complexes.

Agonist stimulation of 7TMRs such as the β 2AR and the V2R also leads to the ubiquitination of the receptors themselves; surprisingly, β -arrestin2 but not β -arrestin1 is required for this process (23, 34). β -Arrestin2 likely acts as an adapter to bring one or more E3 ubiquitin ligases to the activated receptors. For the above 7TMRs, the specific E3 ligases that collaborate with β -arrestin remain to be elucidated. In contrast, the E3 ligases AIP4 and c-Cbl reportedly mediate the ubiquitination of two other mammalian 7TMRs, CXCR4 and PAR2, respectively (35–37). The exact role, if any, of β -arrestin in these systems remains to be determined.

Researchers recently have discovered a further role for β -arrestins in the mediation of ubiquitination and degradation of single-transmembrane receptors, namely the tyrosine kinase IGF-1R and the *Drosophila* Notch receptor. Although β -arrestin was previously reported to act as an endocytic adapter for the IGF1-R, its function in mediating receptor ubiquitination was only recently appreciated. Girnita et al. (38, 39) unexpectedly found that the β -arrestin1 isoform (unlike β -arrestin2, as indicated above for 7TMRs)

acts as an essential adapter to bring the E3 ubiquitin ligase, Mdm2, to the receptor, leading to receptor ubiquitination and degradation. Recently, Mukherjee et al. (40) demonstrated that Kurtz, the unique nonvisual arrestin of *Drosophila*, regulates ubiquitin-dependent downregulation of Notch. Kurtz binds Deltex, a known Notch regulator and a putative E3 ubiquitin ligase for Notch, thus promoting the ubiquitination and degradation of Notch (40). Whether β -arrestin ubiquitination has any role in the signaling mechanisms of these receptors, and whether β -arrestin ubiquitination is even induced by such receptors, remain provocative questions.

Although arrestin homologs have been found only in metazoans, genome sequencing in nonmetazoans has revealed arrestin-like or arrestin domain-containing proteins (41). For *Aspergillus nidulans*, a model fungus, the arrestin-like protein PalF, a component of a fungal ambient pH signaling pathway, becomes phosphorylated and ubiquitinated upon activation of a 7TM receptor, PalH, that responds to alkaline pH. Moreover, PalF phosphorylation and ubiquitination are crucial in linking receptor internalization to the multivesicular body-sorting pathway (42). The pH signaling pathway elicited by the 7TM PalH depends on the arrestin-like protein and its posttranslational modifications but does not require any G protein activity (41). These capabilities of PalF are similar to those of the mammalian β -arrestins, for which phosphorylation and ubiquitination play crucial roles in distinct signaling pathways (see below).

β -ARRESTIN SIGNALING TO THE MITOGEN-ACTIVATED PROTEIN KINASES

In the past seven to eight years, a previously unappreciated function of β -arrestins has come to light: serving as scaffolds for numerous signaling networks. Although we are just beginning to understand some of the signals that β -arrestins regulate, the con-

sequences of these signaling events remain largely a mystery. Below we review current literature relevant to this novel role of β -arrestins as signal transduction scaffolds, with particular attention to the mitogen-activated protein kinases (MAPKs).

Cellular signal transduction involves highly coordinated cascades of events. The number of possible downstream targets for any given member of a signaling network is vast, and to maintain integrity and specificity of signaling, cells employ molecular scaffolds. These are large chaperone complexes that hold together specific members of a signaling network to give them preferential access to one another, thus ensuring the fidelity of a particular signaling response. Recent considerable data show that, in addition to their classic roles in desensitization and internalization, β -arrestins can also act as signaling scaffolds for many pathways and, in particular, those of the MAPKs.

The MAPKs are a family of serine/threonine kinases that include ERK1/2 (also known as p44/p42MAPK), p38 kinases (isoforms α , β , γ , δ), and the c-Jun N-terminal kinases (JNK1, JNK2, JNK3). The downstream effectors of MAPKs control many cellular functions, including cell cycle progression, transcriptional regulation, and apoptosis. ERK1/2 activation exemplifies the prototypical MAPK signaling module: ERK1/2, a MAPK, is phosphorylated by MEK, a MAPK kinase (MAPKK). MEKs are phosphorylated by a variety of Raf isoforms, which are MAP triple kinases, or MAPKKKs. For each of the MAPK family members, this pattern holds true: A MAPKKK activates a MAPKK, which in turn activates a MAPK.

The First Evidence of β -Arrestin Signaling: c-Src recruitment

The first evidence that β -arrestins could act to facilitate signal transduction from 7TMRs came from studies in receptor internalization-defective systems (43–45). These studies reported that dominant-negative versions of

MAPK:

mitogen-activated protein kinase

JNK: cJun

N-terminal kinase

dynamin and β -arrestin1 (45) or chemical blockade of clathrin-mediated internalization (43, 44) diminish receptor signaling to ERK1/2. Although there are a number of ways in which receptors can be internalized, these results suggested that internalization components of at least one of these systems is required for complete MAPK activation under some circumstances.

Shortly thereafter, Luttrell et al. (46) and DeFea et al. (47) discovered that β -arrestin1 can recruit c-Src, a nonreceptor tyrosine kinase family member, to 7TMRs. Src recruitment to the β 2AR results in ERK activation, which can be inhibited by expression of a mutant β -arrestin1 defective in receptor binding or Src binding (46, 48). Src phosphorylation is a necessary step in the activation of various mitogenic signaling pathways activated by 7TMRs, and thus β -arrestins were implicated in carrying cellular signals. Likewise, using the neurokinin-1 receptor and its agonist substance P, DeFea et al. (47) showed that Src recruitment by β -arrestin is necessary for the prevention of apoptosis and propagation of mitogenic signals. The above studies were groundbreaking in introducing the idea of a second wave of 7TMR signaling initiated by β -arrestins.

ERK: The Archetype for β -Arrestin Signaling

Soon after the discovery of β -arrestin-dependent Src recruitment, DeFea et al. (49) and Luttrell et al. (50) showed that β -arrestins scaffold specific components of the MAPK cascade. Using the protease-activated receptor 2 (PAR2), DeFea et al. (47) demonstrated that agonist stimulation results in the formation of a complex containing the activated receptor, β -arrestin1, Raf-1, and phosphorylated ERK. In a similar report using the AT_{1A}R, Luttrell et al. (50) described an agonist-induced β -arrestin2, Raf-1, MEK1, and ERK1/2 signaling complex. This study showed that MEK1 indirectly binds β -arrestin2 through contacts with Raf and ERK, whereas the latter components directly bind β -arrestin. These studies clearly demonstrated that β -arrestins can serve as scaffolding molecules that facilitate cell signaling to ERK. Recently, Scott et al. (51) reported that filamin A, an actin-binding protein, directly binds β -arrestin1 and increases its association with ERK. **Figure 1a** depicts β -arrestin2 scaffolds for ERK activation, as currently understood.

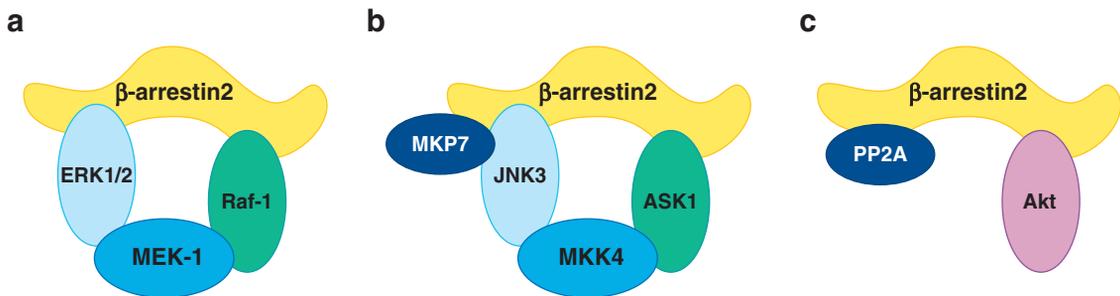


Figure 1

β -Arrestin scaffolds for signaling. (a) A depiction of the β -arrestin scaffold for extracellular signal-related kinase (ERK) activation. ERK1/2 and Raf-1 bind β -arrestin2 directly, and the mitogen-activated protein kinase kinase MEK-1 binds indirectly. It is not known which residues of β -arrestin2 bind ERK and Raf-1. (b) The β -arrestin scaffold for c-Jun N-terminal kinase (JNK) 3 activation. As does ERK, JNK3 and apoptosis signaling kinase 1 (ASK1) bind β -arrestin2 directly, whereas MAP kinase kinase 4 (MKK4) binds indirectly. JNK3 and the negative regulator MAP kinase phosphatase 7 (MKP7) contact the RRS motif (amino acids 195–202). (c) The β -arrestin scaffold for Akt regulation. β -Arrestin2 associates with and is necessary for Akt activation in some systems, although it is not known if Akt and/or PP2A bind directly or indirectly to β -arrestin2.

Once researchers established that β -arrestins were involved in signal transduction to ERK from a variety of 7TMRs, they investigated the functional consequences of these events. One effect of ERK activation is the phosphorylation and activation of Elk-1, an Ets domain-containing transcription factor involved in the transcription of genes that promote cell cycle progression. Using the AT_{1A}R as a model, Tohgo et al. (52) observed that exogenous β -arrestin1 or -2 expression resulted in decreased agonist-stimulated phosphoinositide hydrolysis, yet increased ERK activation. In other words, β -arrestin expression inhibited G protein signaling (through increased desensitization) but increased ERK phosphorylation through a presumably G protein-independent pathway. However, Elk-1-dependent transcription, which typically results from ERK activation, was decreased when β -arrestins were expressed. (52). Confocal microscopy revealed that this effect is a result of the cytosolic retention of ERK by β -arrestins on endocytic vesicles (50, 53), later termed β -arrestin signalosomes (30). Although these studies clarify that β -arrestin-dependent ERK does not have typical nuclear ERK functions, the precise downstream targets activated by β -arrestin-dependent ERK remain unknown. However, cytoskeletal rearrangement and chemotaxis (54–56) have been linked to β -arrestin signaling. In one report, β -arrestin signalosomes were enriched at the leading edge of chemotactic cells, suggesting that β -arrestin-mediated ERK is responsible (55). In accord with this result, Scott et al. (51) showed that β -arrestins, filamin A, and ERK are all necessary for membrane ruffling in Hep2 cells.

RNA silencing technology has provided a powerful tool for distinguishing β -arrestin-dependent activation of ERK from that initiated by G proteins. Previously, studies of β -arrestin signaling generally relied on overexpression of β -arrestins in cell lines, such as COS-7, with very low endogenous β -arrestin expression (50, 53). Small interfering RNAs (siRNAs) allow for the selective elimination of

a particular β -arrestin isoform from an otherwise complete signaling system. Application of siRNA technology to β -arrestin signaling revealed previously unappreciated differences in β -arrestin isoform specificity (57–59). Although siRNA directed toward β -arrestin2 led to a decrease in phosphorylated ERK after stimulation of the AT_{1A}R in HEK-293 cells, β -arrestin1 siRNA resulted in a surprising increase in ERK activation (59). Ren et al. (60) noted a similar pattern for the V2 vasopressin receptor (V2R). This pattern, termed reciprocal regulation, is still not mechanistically understood, nor does it apply to all 7TMRs. For example, some receptors, such as the β 2AR and parathyroid hormone receptor, subtype 1 (PTH1R), exhibit a marked decrease in ERK activation following siRNA depletion of either β -arrestin1 or -2 (61, 62). This pattern may be termed codependent regulation, as both β -arrestins are necessary for ERK signaling. **Table 1** lists the patterns for 7TMRs studied to date.

G protein-mediated and β -arrestin-mediated ERK pathways are both spatially segregated (50, 52, 58) and, according to studies using RNAi, temporally distinct (58) in cells. For the AT_{1A}R studied in HEK-293 cells, G protein-mediated ERK activity was maximal at 2 min after stimulation, with very little contribution to the cellular pool of phospho-ERK after 10 min (58). β -Arrestin2-mediated ERK activity was minimal until 10 min poststimulation but was responsible for nearly 100% of ERK signaling at times beyond 30 min (58). Blockade of G α q-dependent signaling by a protein kinase C (PKC) inhibitor yielded an inverse pattern of ERK phosphorylation when compared with that which resulted from β -arrestin2 siRNA. Interestingly, the two pathways to ERK activation were additive over the entire time course. **Figure 2**, a time-course plot of ERK activation by AngII, depicts these findings.

Mutational analysis supports the independence of G protein and β -arrestin signaling pathways for certain receptors. Mutant

Codependent

regulation: when β -arrestin-mediated signaling from a particular receptor relies on both β -arrestin isoforms and the selective elimination of either isoform eliminates β -arrestin-dependent ERK activation

Reciprocal

regulation: when β -arrestin-mediated signaling from a particular receptor relies upon only one β -arrestin isoform and the other β -arrestin isoform functions to inhibit that signaling

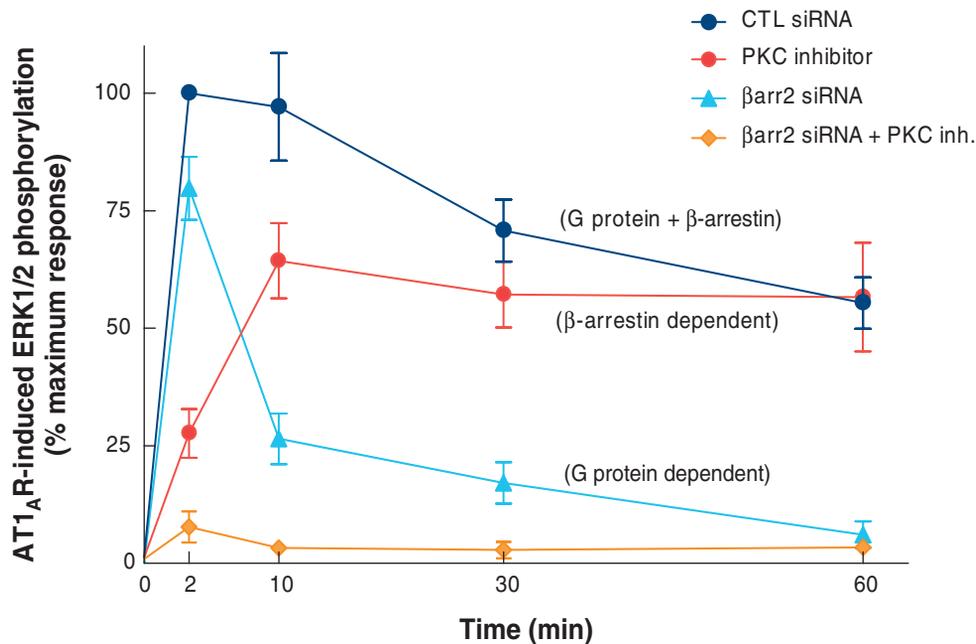


Figure 2

Phospho-ERK activation by the AT_{1A}R is dependent upon β -arrestin2 expression. A time course of ERK phosphorylation stimulated by the AT_{1A}R in HEK-293 cells in response to 100-nM angiotensin II. Cells were transfected with control siRNA or β -arrestin2 siRNA for 72 h and treated with either the PKC inhibitor Ro31-8425 or DMSO for 20 min prior to stimulation. Plots are depicted as a percentage of maximal ERK activation at 2 min in the control siRNA, DMSO-treated sample. Results are averages \pm SEM of five experiments. Figure adapted from Reference 58a, with permission.

receptors that do not couple to their cognate G proteins but still recruit β -arrestins in response to agonist stimulation provide an excellent model with which to study exclusively β -arrestin-mediated signaling. Thus far, researchers have identified such mutants for two receptors, the β 2AR and AT_{1A}R. For the AT_{1A}R, alanine substitution of the first two amino acids of a conserved motif in the second intracellular loop, termed DRY, completely uncouples the receptor from G α q (63) while retaining the capacity to recruit β -arrestins and activate β -arrestin-dependent ERK (64). The ERK that is phosphorylated in response to AngII stimulation of the AT_{1A}R-DRY/AAV receptor depends completely on β -arrestin2 expression. A similar study employed an evolutionary trace method to create a mutant β 2AR (β 2AR^{TYV}) that is uncoupled from G α s but retains the ability to recruit

and signal through β -arrestins (61). Furthermore, β 2AR^{TYV} ERK activation is completely abolished by β -arrestin2 siRNA and is insensitive to protein kinase A (PKA) inhibitors (61).

Biased agonism refers to the preferential activation of one of a number of possible downstream pathways of a receptor by a particular ligand. This concept implies that receptors can exist in an ensemble of distinct conformations in response to ligand binding. For example, in an inactive receptor conformation, G proteins are not dissociated, and β -arrestins are not recruited. Inactive conformations would be expected to predominate in the absence of ligand or upon treatment with an inverse agonist. Conversely, fully active conformations would result from treatment with a full agonist. However, a biased agonist would be expected to elicit a conformational change

Biased agonism:

when a ligand has the ability to preferentially activate one of a number of possible signaling pathways stemming from a particular receptor

that can only recruit β -arrestins without coupling to G proteins, or vice versa.

In light of the two possible pathways of signaling elicited by 7TMRs we describe in this review, G protein or β -arrestin mediated, a biased agonist would specifically activate one of these pathways to a greater extent than the other. A prime example of this bias exists for the AT_{1A}R. A mutational approach was successful in identifying a β -arrestin signaling-specific agonist for this receptor (64, 65), termed SI^{4T8} AngII (SII). Although SII cannot activate G α q signaling, as evidenced by lack of PI hydrolysis (64, 65), calcium mobilization, or diacylglycerol activity, SII can recruit β -arrestin to the AT_{1A}R in a Class B pattern and stimulates ERK in an entirely β -arrestin2-dependent manner (64). Recently, mutation analysis of the AT_{1A}R revealed that specific residues in the seventh transmembrane-spanning domain are necessary for SII mediated signaling but not for AngII signaling. This report suggests that SII can induce a conformational change that is distinct from that induced by AngII (66). In vivo, SII mediates different physiological effects than AngII; i.e., whereas AngII stimulates intake of both water and salt in mice, SII increases only salt consumption (67).

Similarly, for the β 2AR, the compound ICI118551, a well-established inverse agonist for adenylyl cyclase (68), induces ERK phosphorylation that completely depends on β -arrestin2 expression (69). Finally, for the V2R, the inverse agonist SR121463B recruits β -arrestin and stimulates ERK (69). These data further support the independence of G protein and β -arrestin signaling pathways for some 7TMRs.

However, independence of these pathways is not displayed by all receptors. A naturally occurring system of biased agonism exists for the chemokine receptor CCR7: One ligand (termed ELC/CCL19) can activate both G protein and β -arrestin signaling, and another ligand (SLC/CCL21) activates only G protein signaling and does not recruit β -arrestins.

In this system, β -arrestin signaling is also dependent upon G protein activity, as pertussis toxin treatment of cells eliminates all activation of ERK through the CCR7 receptor (70). Other G α i-coupled receptors show a similar pattern. For example, CXCR4 can induce β -arrestin-dependent chemotaxis, which is abolished by pertussis treatment (71). Also, in studies of the AT_{1A}R and lysophosphatidic acid (LPA) receptor in HEK-293 cells, AngII-induced chemotaxis was slightly reduced, and LPA-induced, β -arrestin-dependent chemotaxis was eliminated by pertussis, consistent with the coupling of the LPA receptor to G α i and the coupling of AT_{1A}R to both G α q and G α i for these cellular responses (56).

Although the downstream effectors of β -arrestin signaling to ERK have not been elucidated, a recent report suggests one physiological outcome with implications for Parkinson's disease. Rotenone, a pesticide, is toxic to dopaminergic (DA) neurons and is used as a model of Parkinson's disease. Stimulation of the group III metabotropic glutamate receptor (mGluR_{III}) attenuates the toxicity of rotenone on DA neurons. Jiang et. al. (72) found that the mechanism for this stems from L-AP-4 (an mGluR_{III} agonist) stimulation of ERK that depends on dynamin, β -arrestin2, and c-Src. The β -arrestin-activated ERK stabilizes microtubules, which protects DA neurons from rotenone toxicity (72) and prevents Parkinson's-like symptoms.

β -Arrestin and JNK3 Signaling

Another MAPK activated by β -arrestin signaling pathways is JNK3. Unlike the other two members of this kinase family, JNK1 and JNK2, which are ubiquitously expressed, JNK3 shows tissue specificity, with expression restricted to the brain and to a lesser extent the heart and testis (73). JNK3 was originally identified as a binding partner of β -arrestin2 in a yeast two-hybrid screen, and subsequent investigation revealed that the two upstream kinases, MAP

kinase kinase 4 (MKK4) and apoptosis signaling kinase (ASK1), were also present in the complex (74, 75). Thus, β -arrestin2 scaffolds an entire signaling module for JNK3, as was previously shown for ERK1/2. This module responds to signaling from a 7TMR, the AT1_AR, in a time-dependent fashion correlating with β -arrestin2 recruitment and receptor binding (74). Interestingly, phosphorylated, active JNK3 can only be detected in association with β -arrestin2 and is excluded from the nucleus. A subsequent study of β -arrestin2 subcellular localization determinants confirmed this finding, as a mutant β -arrestin2 lacking the nuclear export signal showed a redistribution of JNK3 to the nucleus (76). These findings closely parallel the situation with ERK, for which the β -arrestin-activated kinase is found only in the cytoplasm.

Recently, Willoughby et al. (77) showed that a fourth partner in this signaling module, the dual-specificity phosphatase MAP kinase phosphatase 7 (MKP7), dynamically interacted with β -arrestin2. MKP7 seems to function as a “reset switch” in the JNK3 cascade, as it rapidly dissociates from β -arrestin2 upon AT1_AR stimulation and later reassociates to dephosphorylate JNK3 in the complex (77). This is the first report of a negative regulator in a β -arrestin signaling module, which raises the possibility that other β -arrestin scaffolds bind their own phosphatases. **Figure 1b** shows a schematic representation of the β -arrestin2 scaffold for JNK3 activation.

p38 Activation via β -Arrestin

The third and final member of the MAPK family, p38, has four isoforms (α , β , γ , δ) and is primarily involved in eliciting a transcriptional response to inflammatory cytokines, growth factors, and cellular stresses. Although researchers have not directly shown that β -arrestins (*a*) scaffold either p38 itself or upstream kinases or (*b*) lead to p38 phosphorylation, three reports have implicated β -arrestins in p38 activation (71, 78, 79). The chemokine

receptor CXCR4-induced chemotactic response, which is β -arrestin dependent, is also sensitive to p38 inhibitors (71). These data suggest that at least some cellular functions regulated by β -arrestin signaling involve p38, even though direct β -arrestin-dependent p38 phosphorylation is not documented.

More direct evidence for p38 signaling comes from work with the cytomegalovirus 7TMR US28, which is a constitutively active chemokine receptor homolog (78). US28 is constitutively phosphorylated by GRKs and recruits β -arrestin in the absence of agonist. When the cytoplasmic tail of US28 is truncated, the constitutive recruitment of β -arrestin is lost, and subsequent p38 activation is diminished (78).

Very recently, Bruchas et al. (79) demonstrated that the kappa opioid receptor activates p38 by a mechanism that involves GRK3 phosphorylation and β -arrestin2. In their study, Bruchas et al. used both GRK3^{-/-} MEFs and siRNA directed against β -arrestin2 in striatal astrocytes. They discovered that activated p38 colocalized with β -arrestin2 in features reminiscent of the signalosomes found with β -arrestins and ERK (79).

β -ARRESTIN SIGNALING TO OTHER KINASES

PI3 Kinase and Antiapoptosis

Phosphatidylinositol 3 kinase (PI3K) is a common signaling intermediate of tyrosine kinase receptors such as the insulin-like growth factor-1 receptor (IGF1-R). PI3K catalyzes the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5 triphosphate (PIP3). IGF1-R stimulation leads to PI3K activity, which promotes cell growth and inhibits apoptosis. In mouse embryo fibroblasts (MEFs) lacking both β -arrestin1 and -2, IGF-1 was unable to stimulate PI3K activity (80). This effect was rescued by expression of exogenous β -arrestin1 and was independent of

the tyrosine kinase activity of the activated IGF1-R (80). This is in contrast to the classical IGF-1-stimulated pathway, whereby agonist binding causes receptor autophosphorylation and subsequent tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), leading to PI3K and Akt activation.

Akt Activation

One important downstream target of PI3K signaling is Akt, also known as protein kinase B (PKB). Akt is pivotal in promoting cell survival and preventing apoptosis through numerous downstream effectors. In the above-mentioned IGF-1-stimulated PI3K study, Akt was phosphorylated in β -arrestin1-expressing cells but not in β -arrestin1/2 knockout cells (80). This β -arrestin1-dependent Akt signaling led to increased protection from apoptosis (80).

Thrombin, through protease-activated receptors (PARs), can activate Akt by two mechanisms, one $G\alpha$ dependent and the other β -arrestin1 dependent (81). The function of β -arrestin1-mediated Akt activation is still unclear, as its inhibition does not affect cell cycle progression (81–83).

Recently, β -arrestin2 has also been implicated in Akt signaling. Using the D2 class of dopamine receptors, Beaulieu and colleagues (84) found that dopamine stimulates the formation of a signaling module consisting of β -arrestin2, Akt, and its negative regulator, protein phosphatase 2A (PP2A) (**Figure 1c**). In mice lacking β -arrestin2, dopamine was unable to regulate Akt signaling, as PP2A and Akt could no longer interact. Furthermore, behavioral effects associated with dopamine administration, such as increased locomotor activity and wall climbing, were lost in these mice, demonstrating the importance of β -arrestin2 in mediating these events (84). This work is another example of a β -arrestin signaling module, much like the JNK3 module, whereby β -arrestin assembles activators and negative regulators of a pathway as a scaffold.

Coordinate Regulation of RhoA by β -Arrestin1 and $G\alpha_q$

Pertinent to its role in chemotaxis (see Reference 8a), β -arrestin signaling can also mediate rearrangement of cytoskeletal components. In particular, $AT1_A$ R stimulation activates the small GTPase RhoA in a β -arrestin1-dependent fashion, leading to the formation of stress fibers (54). Interestingly, this activation of RhoA depends on both $G\alpha_q$ and β -arrestin1 (54), whereas $AT1_A$ R-stimulated, β -arrestin-mediated ERK signaling is independent of G protein activation (64). This implies that the necessity of G protein activity for β -arrestin-mediated functions is specific for a given cellular outcome.

NUCLEAR β -ARRESTIN SIGNALING

β -Arrestin Trafficking and the Nucleus

In addition to the above-mentioned roles that β -arrestins play in cellular signaling in the cytosol, there is new evidence that β -arrestins can also regulate nuclear processes such as transcription of new RNA. Although both β -arrestin1 and -2 contain nuclear localization signals, only β -arrestin2 has a nuclear export signal. As a consequence, β -arrestin1 is present in both the nucleus and cytoplasm, whereas β -arrestin2 is only in the cytoplasm (17). However, upon treatment of cells with leptomycin B, an inhibitor of CRM1-mediated nuclear export, β -arrestin2 accumulates only in the nucleus (76). These data imply functional roles for both β -arrestin1 and -2 in the nucleus.

Transcriptional Regulation by β -Arrestins

Changes in transcriptional responses are a common outcome of signaling via 7TMRs. Although cytoplasmic signaling by β -arrestins has been extensively described, its effects in

the nucleus have not received attention until very recently. One transcription factor family regulated by β -arrestin is NF κ B, a nuclear transcription factor that regulates the expression of genes involved in inflammation, the autoimmune response, cell proliferation, and apoptosis (85, 86). Two independent studies identified β -arrestins as binding partners for I κ B α , a protein that binds NF κ B and inhibits its nuclear translocation and subsequent activity (87, 88). Both β -arrestin isoforms can sequester I κ B α -NF κ B complexes in the cytosol and attenuate NF κ B-dependent nuclear transcription in response to 7TMR or cytokine stimulation. Furthermore, β -arrestin2 can regulate UV-induced NF κ B activity via a pathway involving casein kinase II-mediated I κ B α phosphorylation (89). In this case, UV irradiation of heterologous cells activates casein kinase II to phosphorylate β -arrestin2. This β -arrestin2 phosphorylation reduces the interaction of β -arrestin with I κ B α , causing I κ B α degradation and releasing NF κ B for nuclear entry and transcriptional activity.

Gesty-Palmer et al. (90), using cDNA microarray technology, recently examined transcriptional regulation attributable to β -arrestin2 signaling in response to LPA. ERK signaling that results from LPA stimulation of MEFs was diminished in β -arrestin2 knockout ($^{-/-}$) MEFs. Most distally, of the seven mRNAs upregulated by LPA treatment in wild-type MEFs, only four persisted in β -arrestin2 $^{-/-}$ MEFs. Interestingly, there was an upregulation of several mRNAs in the β -arrestin2 $^{-/-}$ MEFs that was not present in wild-type MEFs. Although the precise mechanism was not defined, these data suggest that β -arrestin signaling has nuclear effects that are both positive and negative with regard to gene expression.

β -Arrestins may also control transcription without ever entering the nucleus by activating nuclear receptors such as the retinoic acid receptor (RAR). The RARs control gene expression in response to extracellular stimuli and are regulated by their phosphorylation state. A recent report found that β -arrestin2

signaling regulates all RAR and RXR receptors studied to some extent. In particular, RAR- β 2-induced transcription in PC12 cells completely depends on β -arrestin2-mediated ERK activity (91). This β -arrestin-dependent transcriptional regulation inhibits cell growth through expression of nerve growth factor.

Kang et al. (92) recently observed a much more direct role of β -arrestins in mRNA transcription. This report found that β -arrestin1—which, unlike β -arrestin2, is localized to the nucleus without the addition of leptomycin B—binds directly to the promoter regions of several genes, including *c-fos* and *p27*. Furthermore, β -arrestin1 recruits p300, the histone acetyltransferase, to these promoters via an interaction with its binding partner CREB (92). This recruitment results in an increase in transcription from the promoters bound by β -arrestin1. Both the amount of β -arrestin1 and the level of transcriptional activity at these promoters can be regulated by 7TMR stimulation, in this case the δ -opioid and κ -opioid receptors. However, not all 7TMRs can initiate this signaling, as the β 2AR and μ -opioid receptor were unable to elicit such a response (92). In summary, these data provide an excellent mechanism for direct β -arrestin1-mediated control of gene expression upon 7TMR stimulation.

ACTIVATION OF β -ARRESTIN: CONFORMATIONAL CHANGES

Most signal transduction mechanisms are guided by ligand-induced conformational changes in the receptor, which are then translated to downstream effectors, resulting in a specific signal output. Plasma membrane translocation of β -arrestin to activated receptors and its signaling capabilities even in the absence of receptor-G protein interaction strongly suggest the existence of specific receptor conformation(s) for β -arrestin binding (61, 64). Moreover, these findings indicate that β -arrestins must undergo their own activation-dependent conformational changes to facilitate downstream

signaling. Further supporting this is evidence that many of the nonreceptor-partner interactions of β -arrestins, such as the formation of MAPK scaffolds, are facilitated upon receptor activation.

Much evidence, including mutagenesis and *in vitro* biochemical and biophysical characterizations, suggests that visual arrestin undergoes substantial conformational changes as it binds to light-activated, phosphorylated rhodopsin (93–95). The X-ray structures of bovine visual arrestin and β -arrestin1 in the basal inactive state indicate that arrestin is an elongated molecule with two domains (N- and C-domain), connected through a 12-residue linker region (96–99). A notable feature is a hydrogen-bonded network of buried, charged side chains (the polar core) embedded between the N- and C-domains at the fulcrum of the β -arrestin molecule (100). Disruption of the polar core by the phosphate moieties on activated receptors and the rearrangement of the “three-element interaction” (i.e., N and C termini and the single α -helix of β -arrestin) are components of a mechanism by which β -arrestin activation and conformational change are proposed to occur. Xiao et al. (101) recently demonstrated that conformational changes of the nonvisual β -arrestin2 occur in the presence of a phosphorylated, 29-mer peptide that corresponds to the carboxyl tail of the V2R. Addition of the V2R phosphopeptide to β -arrestin2 *in vitro* led to the exposure of a buried tryptic cleavage site (arginine 394) as well as the release of residues 371 to 379 in the C terminus of β -arrestin2, which contain the sites for clathrin interaction. In this activated conformation induced by the phosphopeptide, β -arrestin binding to clathrin increased at least tenfold.

In another recent study, Bouvier and colleagues (102) monitored the conformational changes in the β -arrestin2 molecule by utilizing bioluminescence resonance energy transfer (BRET) assays. BRET is a distance-dependent nonradiative energy transfer that occurs when an acceptor fluorophore such as the yellow fluorescent protein (YFP) comes

within 10–100 Å and captures the energy released by the bioluminescent donor enzyme luciferase upon degradation of the latter's substrate (e.g., coelenterazine). Typically, the donor and acceptor molecules are fused to two independent proteins for testing intermolecular interaction by BRET. However, Bouvier's group adapted this principle to study intramolecular BRET by flanking the β -arrestin molecule with a luciferase moiety at the N terminus and YFP at the C terminus (103). Thus, changes occurring in β -arrestin conformation would cause a rearrangement of the two ends of β -arrestin, producing a BRET signal. Indeed, such intramolecular BRET signals occurred in the luciferase- β -arrestin2-YFP protein upon activation of various 7TMRs. However, the half-life of V2R-stimulated intramolecular BRET in β -arrestin2 (~5 min) was significantly slower than the kinetics of β -arrestin recruitment to the V2R (~0.8 min). Furthermore, quantitatively similar BRET signals were produced in both wild-type β -arrestin and the β -arrestin mutant R169E, which binds nonphosphorylated receptors (104, 105). Accordingly, the authors concluded that the conformational changes in β -arrestin (represented by intramolecular BRET) likely occur due to the binding of β -arrestin-interacting proteins subsequent to the binding of β -arrestin to the receptors and are not due to β -arrestin's interaction with the phosphorylated domains of the 7TMR. However, this conclusion is debatable for at least two reasons. First, the chimeric protein may have constraints that restrict release of the C-terminal region. Hence, this technique may not discern the conformational changes that occur upon binding receptor phosphates. Second, although quantitatively both the wild-type and mutant proteins display similar BRET responses, the wild type may undergo conformational rearrangements qualitatively different than the mutant, wherein the basal constraints in the polar core are absent. The BRET technique cannot distinguish between such qualitative differences.

Thus, receptor binding may activate β -arrestin, causing multiple, parallel, or stepwise conformational changes to induce downstream signaling pathways. Additional work will be necessary to determine whether the β -arrestin conformational changes induced by 7TMR binding are merely a means to facilitate binding of nonreceptor partners, or a process that initiates β -arrestin-mediated signaling pathways such as those leading to MAPK activation, or both.

NEW RECEPTORS FOR β -ARRESTIN

Parallel to the discoveries of the participation of β -arrestin in various signaling pathways has been the tantalizing finding that β -arrestin can bind receptors that are structurally unrelated to 7TMRs. Thus, β -arrestins regulate signaling and/or endocytosis of IGF1R, Frizzled, smoothened, TGF β RIII, LDLR, Na⁺/H⁺ exchanger NHE5, Toll-like receptor, Interleukin1 receptor, and *Drosophila* Notch (40, 44, 106–111). Accordingly, the biological roles of β -arrestin in signal transduction are likely much broader than we currently appreciate. Because this subject is beyond the scope of this review, these capacities of β -arrestins are not discussed here.

UNANSWERED QUESTIONS AND EMERGING AREAS IN β -ARRESTIN SIGNALING

Dimerization of β -Arrestins

Although homo- and heterodimerization of heptahelical receptors may have important pharmacological and functional implications (112, 113), very few reports have addressed this phenomenon for the non-visual β -arrestins. Purified inactive full-length β -arrestin1 (418 residues) and β -arrestin1–393, a truncation mutant, exist as monomers, according to gel-filtration chromatography (99). However, another truncation mutant, β -arrestin1–382, exists as a mix-

ture of monomeric and dimeric species. Interestingly, this mutant was previously identified as a β -arrestin species that binds unphosphorylated β 2AR reconstituted in liposomes. This phosphorylation-independent mutant was termed constitutively active because it could desensitize receptors in *Xenopus* oocytes even in the absence of GRK-mediated phosphorylation (105). Full-length β -arrestin1 forms only monomeric crystals, whereas the truncated β -arrestin1 (1–382) forms crystals containing exclusively dimers. This scenario is opposite to that observed in visual arrestin, which in solution exists as a tetramer at high concentrations (200 μ M) and as mixed monomer-dimer species at lower concentrations. Because arrestin is highly abundant in rod outer segments, it may oligomerize under inactive physiological conditions. In contrast, arrestin-rhodopsin binding occurs at low concentrations of arrestin, suggesting that monomeric forms of arrestin predominating at low concentrations actually can be active.

Researchers have recently implicated phosphoinositides in arrestin oligomerization. Phosphoinositides can function as important modulators of light-dependent trafficking of *Drosophila* visual arrestin (114–116). For mammalian β -arrestins, the soluble inositol hexakisphosphate (IP6) displays tighter binding than the membrane-associated phosphoinositides PIP2 and PIP3 (117, 118). Gaidarov et al. (117) mapped a high-affinity phosphoinositide-binding site located on the C-domain of β -arrestin2. Although mutation of this site did not ablate clathrin binding or membrane translocation of β -arrestin, it prevented localization of β -arrestin in clathrin-coated pits, thus impairing the endocytic properties of β -arrestin. Milano et al. (118) recently cocrystallized β -arrestin1 with IP6 in the basal state and identified an additional low-affinity IP6-binding site. Interestingly, IP6 mediated an interaction between the N-domain of one β -arrestin1 molecule with the C-domain of a second β -arrestin1

molecule, thus forming a head-to-tail symmetry of β -arrestin in the crystal lattice. Mutation of the basic residue patches forming the IP6 contact sites prevented the formation of β -arrestin1 oligomers while increasing the propensity for nuclear localization. These mutants, defective in both IP6 binding and oligomerization, nevertheless bound nonreceptor partners such as clathrin, AP2, and ERK2. Benovic and colleagues (118) hypothesize that the modulation of IP6 levels in the cytosol regulates the oligomerization state of β -arrestin, probably by multiple complex pathways. Cellular IP6 locks β -arrestin in an inactive oligomeric complex, which is a regulatory step that limits plasma membrane and nuclear localization of β -arrestin. Marullo's group (119) showed that homo- and hetero-oligomerization of exogenously expressed β -arrestins occur constitutively in mammalian cells. Cellular expression of a β -arrestin2-FKBP-GFP molecule, containing a FKBP-binding motif, and induction of its oligomerization by the FKBP-dimerizing small molecule AP20187 did not interfere with its trafficking profile with AT_{1A}Rs. Thus, although IP6-induced oligomers form inactive cytosolic pools, oligomerization per se may not interfere with the classical functions of β -arrestin. Whether activated β -arrestins that form signalosomes upon binding 7TMRs are homo- or heterodimers or even monomers currently remains an unsolved question.

Ubiquitination, a Possible Mechanism that Links the Endocytic and Signaling Properties of β -Arrestin

Agonist-stimulated β -arrestin ubiquitination not only regulates receptor internalization and trafficking of receptor-arrestin complexes to the endosomal compartments but also appears important for stabilizing ERK activity on endosomes. A lysine doublet (residues 11, 12) in β -arrestin2 functions as a crucial site for stable ubiquitination in response to an-

giotensin stimulation and is required for endosomal trafficking as well as for scaffolding phosphorylated ERK in these compartments (25). However, ubiquitination evoked by other Class B receptors, such as the V2R and the neurokinin1 receptor, proceeds at alternate lysine residues, suggesting heterogeneity of the modified receptor-bound β -arrestin. This presents a very provocative scenario in which a specific receptor can provoke ubiquitination at specific site(s), specific to that receptor- β -arrestin pair. These ubiquitination patterns may correspond to particular β -arrestin conformations induced upon receptor binding that expose specific lysine residues for ubiquitination, thus allowing for receptor-specific signaling pathways mediated by β -arrestin. Unlike in phosphorylation, the attachment of ubiquitin adds tertiary structure to the substrate protein, allowing larger conformational changes. Polyubiquitin chains (120), such as those formed on β -arrestin, may specify further conformational complexities and present a foundation for the binding of the many downstream endocytic and/or signaling partners of β -arrestin.

Reciprocal Versus Codependent Regulation of β -Arrestin-Dependent ERK

One of the most intriguing findings regarding β -arrestin signaling is the issue of isoform specificity. Data from siRNA experiments show two distinct patterns for β -arrestin-mediated ERK from 7TMRs. Some receptors, such as the β 2AR, depend on both β -arrestins 1 and 2 for G protein-independent ERK, as a loss of either inhibits this branch of signaling (61). In this review, we term this pattern codependent β -arrestin-ERK, as both β -arrestins are necessary and neither one is sufficient by itself. In contrast, β -arrestin-dependent ERK activated by the AT_{1A}R depends exclusively on β -arrestin2 expression. When β -arrestin1 expression is silenced, G protein-independent ERK increases,

a phenomenon referred to as reciprocal regulation (59). The opposite is true for PAR1: β -Arrestin1 facilitates signaling to ERK, whereas silencing of β -arrestin2 increases β -arrestin1-dependent ERK (121). **Table 1** classifies various receptors as reciprocal or codependent with regard to β -arrestin signaling.

The molecular mechanisms governing this reciprocal regulation have not been elucidated; however, rescue experiments provide some insight. In **Figure 3**, either control siRNA or human β -arrestin2 siRNA is used in conjunction with expression of a rat β -arrestin2-GFP fusion protein, which is insensitive to silencing by virtue of sequence differences between the species. Expression of rat β -arrestin2 can restore >90% of the ERK activity that is eliminated by β -arrestin2 siRNA (**Figure 3**). Surprisingly, rat β -arrestin1 can also rescue approximately 15% of the ERK activity (S. Ahn & R.J. Lefkowitz, unpublished data). This suggests that β -arrestin1 can function as an ERK scaffold, albeit to a much lesser extent than β -arrestin2. In response to AngII, both β -arrestins normally are recruited to the receptor robustly and equally well. In this experiment, β -arrestin1 siRNA would be predicted to increase β -arrestin2 recruitment through loss of competition and thus increase β -arrestin2-dependent ERK signaling (59). Although this portrayal explains reciprocal regulation, it does not address why receptors such as the β 2AR and AT1_AR display such different patterns in the same cell type, HEK-293. One possible explanation for these findings is preferential recruitment of β -arrestin homo- or hetero-oligomers, a subject worthy of future investigation.

Receptor Phosphorylation and β -Arrestin Signaling

Another puzzling question is the extent to which receptor phosphorylation is necessary for, or contributes to, β -arrestin signaling pathways. Here again, the data show two opposite patterns, and the clearest example of

each comes from the β 2AR and AT1_AR. For both these cases, a mutant receptor that is unable to be phosphorylated on the cytoplasmic tail (by either alanine substitution or truncation of all serines and threonines) is available. In **Figure 4**, the β 2AR, which lacks both GRK and PKA phosphorylation sites (β 2AR^{GRK-,PKA-}) and which cannot recruit β -arrestins (61), also cannot mediate β -arrestin-dependent ERK activation. This is expected because GRK phosphorylation typically precedes β -arrestin binding to the receptor. However, in the case of the AT1_AR, the corresponding mutant (AT1_AR ^{Δ 324}) (122) can elicit the same amount of β -arrestin-dependent ERK as the wild-type receptor (**Figure 4**). Whereas the mutant β 2AR cannot recruit β -arrestin at all following stimulation, the mutant AT1_AR can, although it does so in a much weaker Class A pattern than the wild-type Class B receptor (**Table 1**). Taken together, these data suggest that the recruitment of β -arrestin to the plasma membrane, even in a transient Class A pattern, is sufficient to induce β -arrestin-mediated signaling to ERK. A recent study supporting this theory used an artificial system of β -arrestin translocation (103). In the absence of receptor stimulation, β -arrestin recruitment to the membrane was sufficient to trigger ERK activation (103).

However, another report found that mutation of some of the phosphorylation sites in the cytoplasmic tail of Orexin-1 receptor resulted in the loss of sustained ERK activation, yet this mutant still retained some ability to recruit β -arrestins (123). Similarly, cytoplasmic tail truncation mutants of the PAR2 receptor that retained the ability to recruit transiently (Class A), but not stably associate with, β -arrestins lost the ability to activate a prolonged ERK response (124). In another example, using a system of ligand bias for the CCR7 receptor, Kohout et al. (70) found that ELC (CCL19), the ligand capable of inducing receptor phosphorylation, led to an approximate fourfold increase in ERK activation as compared with SLC (CCL21), the ligand that

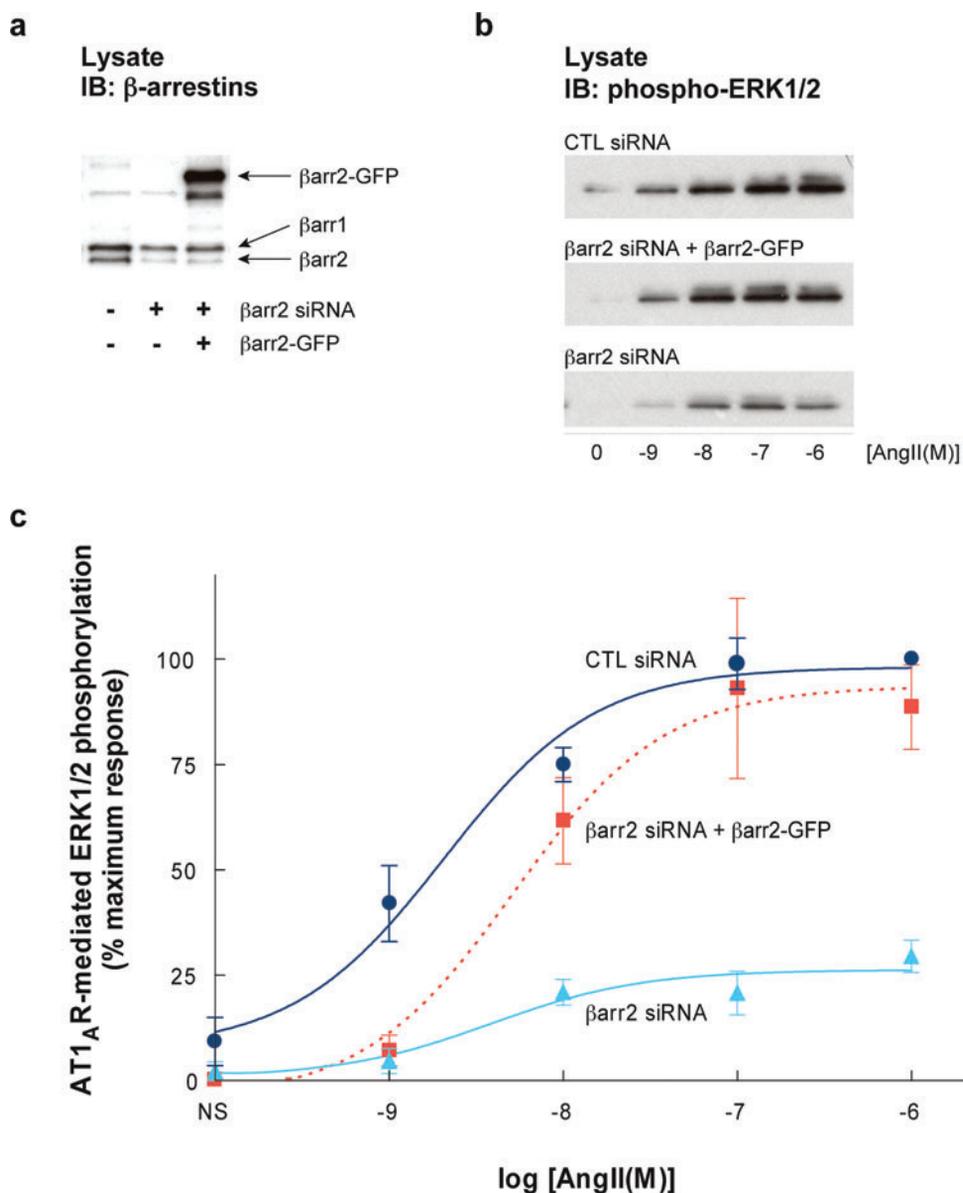


Figure 3

Nonsilenced rat β -arrestin2 (β arr2) expression rescues human β -arrestin2 siRNA effects on ERK activation in HEK-293 cells. Results are averages \pm SEM from five experiments. Cells were treated with either control siRNA (CTL) and the appropriate amount of vector DNA, human β -arrestin2 siRNA and vector DNA, or human β -arrestin2 siRNA and the expression vector for rat β -arrestin2-GFP. Seventy-two hours posttransfection, cells were stimulated with the indicated doses of AngII and analyzed for phospho-ERK. (a) Western blot with an antibody specific for β -arrestins. (b) Representative phospho-ERK western blots. (c) Dose response curve of averaged data for ERK phosphorylation by the AT_{1A}R taken at 5 min poststimulation in HEK-293 cells. IB denotes immunoblot.

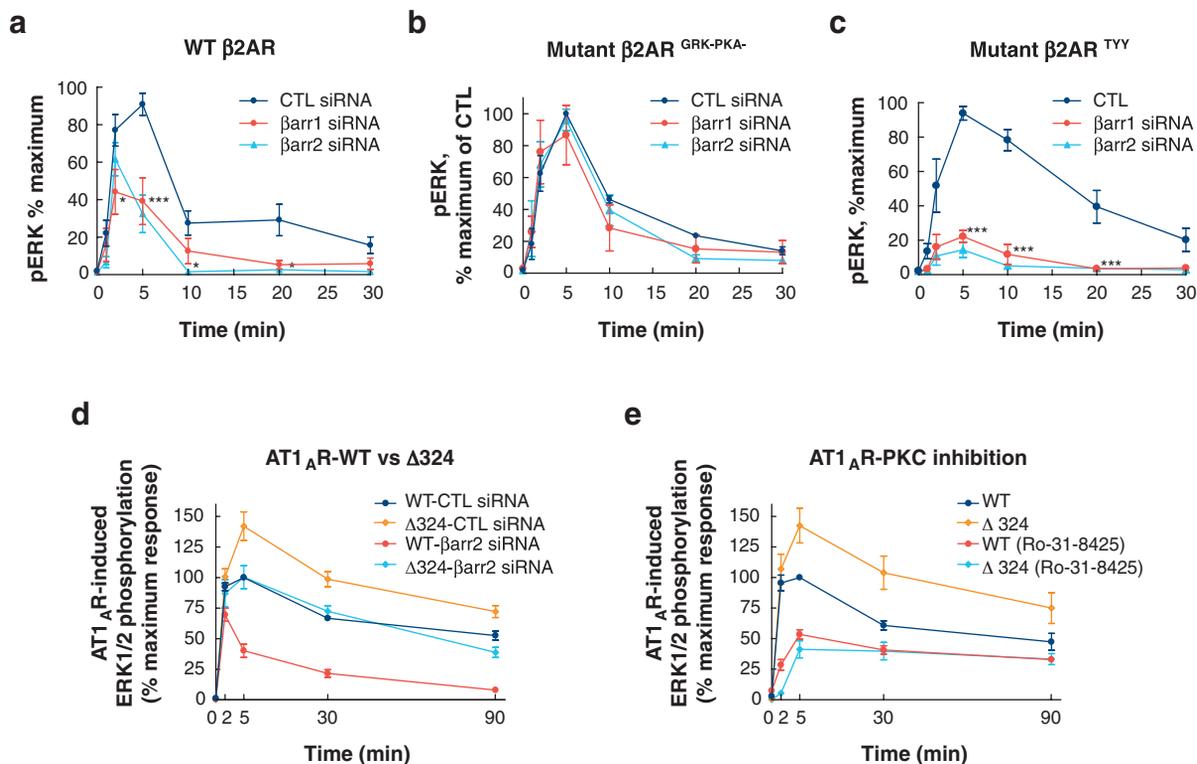


Figure 4

Phosphorylation of receptors is required for β -arrestin signaling for the β -2 adrenergic receptor (β 2AR) but not for the angiotensin II receptor type 1A (AT1_AR). For *a*, *b*, and *c*, plots represent the average of four to six experiments \pm SEM. *a* and *c* are reproduced from Reference 61, with permission. WT denotes wild type. (*a*) A time course of phospho-ERK activation by the transfected human β 2AR in HEK-293 cells in response to 100-nM isoproterenol stimulation. Results were normalized to 100% as control siRNA at 5 min. Cells were transfected with receptor plus control siRNA (CTL siRNA), β -arrestin1 siRNA (β arr1 siRNA) or β -arrestin2 siRNA (β arr2 siRNA). (*b*) The same experiment as in *a* but performed with the GRK⁻, PKA⁻ mutant human β 2AR, which has 14 serines and threonines mutated to alanine and thus cannot be phosphorylated by PKA or GRKs. (*c*) The same experiment as in *a* but performed with the TYY mutant human β 2AR (which can only signal through β -arrestins and cannot couple to G proteins). Adapted from Reference 61, with permission. (*d*) Time course of ERK activation with 100-nM AngII as previously described for the AT1_AR or cytoplasmic tail truncation mutant, AT1_AR- Δ 324 (which cannot be phosphorylated by PKC or GRKs), with either control siRNA (CTL siRNA) or β -arrestin2 siRNA (β arr2 siRNA). Results depicted are averages \pm SEM for nine experiments. (*e*) The effect of PKC inhibition of the AT1_AR and truncation mutant, AT1_AR- Δ 324. The PKC inhibitor, Ro31-8425, at 1 μ M or equivalent volume of DMSO was used with 20-min pretreatment. Results depicted are averages \pm SEM for nine experiments.

cannot recruit β -arrestins. Mutants of CCR7 that could not be phosphorylated maximally also did not activate ERK maximally (70). These results of Kohout et al. show a strong correlation between receptor phosphorylation and β -arrestin-mediated ERK activation. However, in this ELC and SLC system, differ-

ences between β -arrestin recruitment and receptor phosphorylation cannot be addressed because SLC (CCL21) did not recruit β -arrestins, nor were receptor phosphorylation-site mutants tested for β -arrestin recruitment.

In summary, for some receptors such as the β 2AR, Orexin-1, PAR2, and possibly CCR7,

receptor phosphorylation seems necessary for β -arrestin-mediated ERK activation. However, for the AT_{1A}R, phosphorylation on the cytoplasmic tail is dispensable for this signaling: The mechanism we propose is that β -arrestin recruitment, even in a transient Class A pattern, is sufficient for β -arrestin-dependent ERK activation. This mechanism is consistent with all the above data except those for the Orexin-1 and PAR-2 receptors, both of which can still recruit β -arrestins in the absence of phosphorylation yet cannot activate β -arrestin-dependent ERK.

CONCLUDING REMARKS

Researchers have identified numerous substrates, including ERK, JNK3, p38, PI3K, Akt, and RhoA, for β -arrestin-dependent signaling in the cytoplasm. Although the specific events leading to the agonist-induced activation of these signaling pathways have been vastly explored, their downstream targets and physiological outcomes are still largely a mystery. In vivo studies have revealed some expected roles of β -arrestins in receptor desensitization (15, 125, 126), but more recently, some manifestations of the signaling capacities of β -arrestins have also been appreciated (84, 127). These findings reveal the consequences of β -arrestin signaling in complex behavioral phenotypes, thus underscoring the importance of this novel signaling mechanism.

Nuclear β -arrestin signaling is an area of great potential for future research. Although β -arrestin-dependent signaling to ERK does not lead to transcriptional responses through

ERK-dependent factors such as Elk-1 (52), the interaction of β -arrestins and components of the NF κ B pathway can affect transcription negatively (87, 88). Kang et al. (92) recently discovered that β -arrestins can regulate transcriptional changes, and the interaction of β -arrestin1 and p300 provides a promising direct mechanism for activation. Further work is needed to explain β -arrestin1 recruitment to promoters, which may occur through interaction with specific transcription factors.

More tantalizing prospects for the discovery of β -arrestin signaling-dependent physiology include the creation of mice with mutant receptors that perform exclusively β -arrestin signaling. For example, mice expressing the AT_{1A}R-DRY/AAY could be created in the context of the AT_{1A}R knockout mouse (128) and stimulated with AngII. By measuring the typical AngII-stimulated physiology, one could determine how much of the response is carried out by β -arrestin signaling versus G α q. Similarly, one also could take advantage of the available biased agonist for the AT_{1A}R, SII.

β -Arrestin signaling represents a new paradigm in cell biology and potentially a new host of therapeutic targets for diseases of 7TMR dysregulation. Although classic pharmacological solutions have focused exclusively on receptor blockade and G protein-mediated responses, β -arrestins, owing to the only very recent appreciation of their signaling capacities, remain an untapped resource. Future research into the intricate β -arrestin-mediated signaling pathways will shed light on how their modulation can impact human health.

SUMMARY POINTS

1. β -Arrestins not only desensitize G protein-dependent signal pathways but independently promote novel pathways of signal transduction.
2. β -Arrestins serve as scaffolds for the activation of a number of signaling pathways, including ERK, JNK, p38, and Akt.
3. β -Arrestins are posttranslationally regulated by phosphorylation and ubiquitination.

4. Receptor binding induces conformational changes in β -arrestin that may facilitate its scaffolding and signaling functions.
5. Investigators have recently discovered nuclear functions of β -arrestins in transcriptional regulation.
6. β -Arrestins can traffic to receptors other than 7TMRs.

FUTURE ISSUES

1. How do conformational changes in β -arrestin contribute to its signaling and endocytic functions?
2. To what extent do homo- and hetero-oligomerization of β -arrestin affect its ability to desensitize, internalize, and carry out cell signaling?
3. Does ubiquitination of β -arrestin connect its trafficking and signaling roles?
4. What are the receptor-specific factors that determine if receptor phosphorylation is necessary for β -arrestin-mediated ERK activation?
5. Why do some receptors, such as the β 2AR, require both β -arrestin isoforms for ERK activation, whereas others, such as the AT1_AR, only require one?
6. What are the downstream physiological effects of β -arrestin-dependent signaling?

ACKNOWLEDGMENTS

R.J.L. is an investigator with the Howard Hughes Medical Institute (HHMI). S.M.D. and S.A. are supported by HHMI. We acknowledge grant support from the National Institutes of Health (HL 16037 and HL 70631 to R.J.L. and HL080525 to S.K.S.) and the American Heart Association (0530014N to S.K.S.). We thank Donna Addison and Elizabeth Hall for excellent secretarial assistance. We also thank Chris Nelson, Kunhong Xiao, and Kelly Nobles for critical reading of this review.

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