CHAPTER FIVE

Arrestins as Regulators of Kinases and Phosphatases

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Abstract

The discovery that, in addition to mediating G protein-coupled receptor (GPCR) desensitization and endocytosis, arrestins bind to diverse catalytically active nonreceptor proteins and act as ligand-regulated signaling scaffolds led to a paradigm shift in the study of GPCR signal transduction. Research over the past decade has solidified the concept that arrestins confer novel GPCR-signaling capacity by recruiting protein and lipid kinase, phosphatase, phosphodiesterase, and ubiquitin ligase activity into receptor-based multiprotein "signalsome" complexes. Signalsomes regulate downstream pathways controlled by Src

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family nonreceptor tyrosine kinases, mitogen-activated protein kinases, protein kinase B (AKT), glycogen synthase kinase 3, protein phosphatase 2A, nuclear factor-κB, and several others, imposing spatial and temporal control on their function. While many arrestin-bound kinases and phosphatases are involved in the control of cytoskeletal rearrangement, vesicle endocytosis, exocytosis, and cell migration, other signals reach into the nucleus, affecting cell proliferation, apoptosis, and survival. Indeed, the kinase/phosphatase network regulated by arrestins may be fully as diverse as that regulated by heterotrimeric G proteins.

1. INTRODUCTION

By the mid-1990s, the central roles of visual and nonvisual arrestins in G protein-coupled receptor (GPCR) desensitization were well understood.[1] The at-the-time startling discovery that arrestins, like the heterotrimeric G proteins, functioned as discrete GPCR effectors arose from two lines of research. The first was the study of the mechanisms of GPCR endocytosis, which led to the discovery that upon binding to activated receptors, the two nonvisual arrestins, arrestin2 and 3 (β-arrestin1 and 2), act as adapter proteins linking the receptor to components of the clathrin-dependent endocytic machinery.[2,3] The second line of research involved the study of the mechanisms by which GPCRs regulated the nonreceptor tyrosine protein kinase c-Src and the small GTPase Ras pathway.[4,5] The realization that nonvisual arrestins form relatively stable arrestin–GPCR complexes that persist over a timescale of minutes to hours as receptors transit the endocytic vesicle compartment, combined with the observation that arrestin2 bound directly to activated c-Src and recruited it into a multiprotein "signalsome" complex with the receptor,[6] evolved into what has amounted to a reenvisioning of GPCR signal transduction. Rather than functioning solely as ligand-activated guanine nucleotide exchange factors for heterotrimeric G protein, GPCRs have become multifunctional signaling platforms that transmit "pluridimensional" intracellular signals via both G protein and non-G protein effectors.[7] And rather than serving simply as terminators of receptor–G protein coupling, arrestins have become versatile signaling platforms whose binding confers additional GPCR-signaling capability.[8–10]

It is now known that arrestins function as ligand-regulated scaffolds, bringing a host of arrestin-bound proteins to agonist-occupied GPCRs, among them Src family nonreceptor tyrosine kinases[4,11,12]; components

of the extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK)3 mitogen-activated protein kinase (MAPK) cascades[13–15]; the E3 ubiquitin ligase; Mdm2[16]; the cAMP phosphodiesterases, PDE4D3/5[17]; diacylglycerol kinase (DGK)[18]; the inhibitor of nuclear factor-κB, IκBα[19]; the Ral-GDP dissociation stimulator, Ral-GDS[20]; the actin filament-severing protein, cofilin[21]; and the Ser/Thr protein phosphatase 2A (PP2A).[22,23] Indeed, the signaling repertoire accessible to GPCRs through arrestin "coupling" may be as diverse as that mediated by heterotrimeric G -protein-regulated effectors (Fig. 5.1). Moreover, since arrestins uncouple

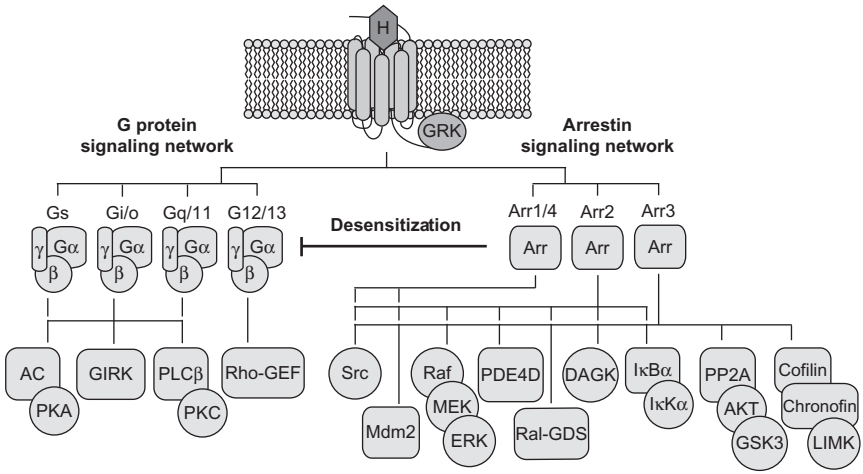

Figure 5.1 Pluridimensional GPCR-signaling networks. Agonist binding to a GPCR stimulates the intrinsic guanine nucleotide exchange factor activity of the receptor, activating heterotrimeric G proteins and initiating second messenger-dependent pathways, including Gs-adenylyl cyclase (AC)–PKA and phospholipase Cβ (PLCβ)–PKC. Numerous other effectors, including gated inwardly rectifying K+ channels (GIRK) and small GTPases like Rho-guanine nucleotide exchange factor (Rho-GEF) are regulated by activated Gα or Gβγ subunits. Agonist-occupied GPCRs are phosphorylated by GRKs, promoting arrestin binding. Arrestins uncouple the receptor and G protein, leading to desensitization of G protein signaling and internalization of the receptor. Arrestins also function as ligand-regulated scaffolds, recruiting catalytic proteins to initiate a second wave of signaling events. Arrestin-coupled effectors include Src family tyrosine kinases (Src), E3 ubiquitin ligases (Mdm2), components of the ERK1/2 mitogen-activated protein kinase cascade (Raf–MEK–ERK1/2), cAMP phosphodiesterases (PDE4D), the Ral-GDP dissociation stimulator (Ral-GDS), diacylglycerol kinases (DAGK), regulators of nuclear factor-κB signaling (IκBα–IκKα), the glycogen synthase kinase 3 regulatory complex (PP2A–Akt–GSK3), and the actin filament-severing complex (cofilin–chronofin–LIMK). Evidence suggests that the signaling network mediated by arrestins may rival the G protein-signaling network in diversity. *Reproduced from Ref. 24.*

the receptor and G protein, arrestin binding can be viewed as the dividing line between two mutually exclusive and temporally discrete GPCR-signaling states.

The first discovered, and still the most diverse, of the arrestin-signaling functions is the regulation of protein and lipid phosphorylation. This chapter discusses the characteristics of arrestins that enable them to function as adapters and scaffolds for protein kinases, their roles as both positive and negative regulators of kinase activity, and the numerous protein and lipid kinase, and protein phosphatase effectors controlled by arrestins. Subsequent chapters in this volume cover the other major classes of arrestin-regulated effectors, including small G proteins, ubiquitin ligases, and regulators of cytoskeletal dynamics. How these signals affect physiological function in embryologic development, intermediary metabolism, vision, cancer, bone, and the cardiovascular, immune, and central nervous systems are then discussed separately.

2. ARRESTINS AS GPCR EFFECTORS

The fundamental duality of arrestin function is well illustrated by a simple experiment. When angiotensin II-stimulated phosphatidylinositol production was assayed in COS-7 cells expressing the angiotensin AT_{1A} receptor, overexpression of either arrestin2 or 3 led to a reduction in the maximal response, an entirely predictable effect of a protein known to promote uncoupling of AT_{1A} receptors from the Gq/11–phospholipase Cβ effector pathway. Yet when angiotensin II-stimulated ERK1/2 activation was assayed under identical conditions, arrestin overexpression led to a paradoxical increase in the response.[25] Such a result could not be a reflection of arrestin-dependent termination of G protein signaling, but rather suggested that the arrestin itself was somehow coupling the AT_{1A} receptor to the ERK1/2 cascade.

2.1. Adaptors or scaffolds?

The nonvisual arrestins clearly function as adapter proteins in the context of clathrin-dependent GPCR endocytosis, binding to agonist-bound GPCRs while at the same time engaging clathrin and the β2-adaptin subunit of the AP-2 complex.[2,3] To transmit signals, in many cases they also function as scaffolds. Scaffolding proteins perform at least three functions in cells: to increase the efficiency of signaling between successive components of an enzymatic cascade, ensure signaling fidelity by dampening cross talk

between parallel cascades, and localize signaling to specific subcellular locations.[26]

The prototypic MAP kinase scaffold is the *Saccharomyces cerevisiae* protein Ste5p.[27] In the yeast pheromone mating pathway, Ste5p binds to each of the three components of the yeast MAP kinase pathway, Ste11p, Ste7p, and either Fus3 or Kss1. Binding of mating factor to the pheromone receptor, a GPCR, leads to translocation of Ste5 to the plasma membrane and activation of the Fus3/Kss1 cascade. Deletion of the *Ste5* gene disrupts the yeast mating process, indicating the essential role of this scaffold in MAP kinase regulation. While there are no structural homologues of Ste5 in mammalian cells, arrestins perform an analogous function, organizing individual pathway components to increase efficiency and fidelity of signaling, imposing spatial constraints, and, importantly, providing for pathway regulation by extracellular signals detected via GPCRs on the plasma membrane.

This scaffolding property is illustrated by the effect of arrestin3 on the activation state of c-Jun N-terminal kinase 3 (JNK3). JNK3 is a neuronal MAPK that is regulated by a phosphorylation cascade composed of the upstream MAPK kinase kinase, Ask1; the MAPK kinase, MKK4; and JNK3. Arrestin3 binds the three component kinases, increasing the efficiency of phosphorylation. Ectopic expression of Ask1 and JNK3 in COS-7 cells is not sufficient to activate JNK3, but simultaneous expression of arrestin3 with Ask1 and JNK3 results in dramatic activation.[13] Arrestin3 is excluded from the nucleus by the presence of a classical leucine-rich nuclear export signal in its C-terminus that is absent in arrestin2.[28,29] Consequently, expression of arrestin3 also leads to a dramatic redistribution of JNK3 from the nucleus into an arrestin-bound cytosolic pool.[13,30]

Arrestin-dependent regulation of the ERK1/2 MAPK cascade provided another early example of arrestin scaffolding. Both arrestin2 and 3 can assemble a complex composed of c-Raf1, MEK1/2, and ERK1/2, and enhance ERK1/2 activation in response to stimulation of protease-activated receptor 2 (PAR2) and angiotensin II AT_{1A} receptors.[14,15,25,31] Agonist binding induces the assembly of a protein complex containing the internalized receptor, arrestin, and activated ERK1/2. This arrestin-mediated signal is independent of heterotrimeric G protein activation, as it can be produced by mutated AT_{1A} receptors that lack G protein-coupling efficacy and by "biased" AT_{1A} receptor agonists that promote arrestin recruitment and receptor internalization without G protein activation.[32] Because the complex formed between PAR2 and AT_{1A} receptors and arrestins is stable, these signalsomes localize activated ERK1/2 to early endosomes, where it remains

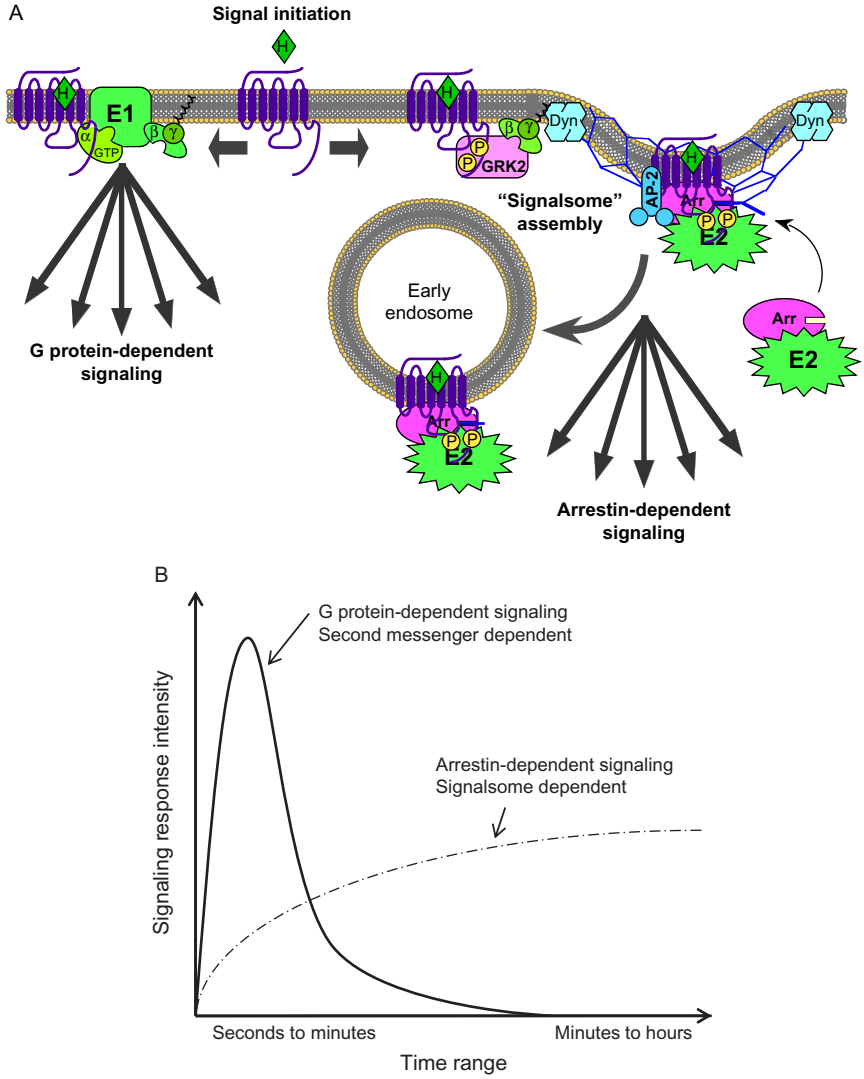

Figure 5.2 Arrestin scaffolds impose spatial and temporal regulation of signaling pathways. (A) Upon agonist (H) binding, GPCRs engage heterotrimeric G proteins, activating G protein-regulated effectors (E1) at the plasma membrane. Within seconds, GRK phosphorylation of the activated receptor creates high-affinity arrestin-binding sites. Arrestin binding uncouples the receptor from heterotrimeric G proteins while targeting it for endocytosis. As arrestins translocate to the receptor, they recruit additional catalytically active proteins (E2) into receptor–arrestin signalsomes. These stoichiometric signaling complexes transmit a distinct set of signals as the receptor internalizes and transits the intracellular compartment. (B) G protein-dependent signaling is characterized by rapid onset followed by waning intensity, reflecting desensitization due to receptor phosphorylation by second messenger-dependent protein kinases and GRKs, and arrestin binding. In contrast, arrestin-mediated signals are of slower onset and often sustained in duration. *(A) Reproduced from Ref. 33.*

associated with the receptor–arrestin complex[14,15] (Fig. 5.2A). In fact, an estimated 75–80% of the active ERK1/2 produced in response to short-term stimulation of the PAR2 receptors is associated with the GPCR–arrestin signalsome.[14] As a result, nuclear translocation of active ERK1/2 is retarded and its kinase activity is directed away from nuclear, and toward cytosolic, targets.[31,34,35]

2.2. Mechanism of arrestin-signaling scaffolds

In contrast to the clathrin and AP-2 binding sites in the C-terminus, and receptor recognition motifs within the N- and C-terminal globular domains of arrestin (see Chapter 3), the binding sites for most arrestin-regulated signaling partners have not been precisely mapped. In fact, all three components of the c-Raf1–MEK1–ERK1/2 and ASK1–MKK4–JNK3 cascades can bind to separately expressed arrestin N- and C-domains, suggesting that they each make multiple contacts across the exposed cytosolic face of the receptor-bound arrestin.[36,37]

Since arrestins undergo a conformational change when they bind to GPCRs, activation of arrestin-bound effectors might then proceed by either of two mechanisms. Receptor binding might change the affinity of arrestin for its nonreceptor partners, allowing the signalsome complex to form only on the receptor. Alternatively, the arrestin–effector complex may be preformed, with activation resulting either from conformational changes in the arrestin that provide the proper orientation of pathway components or from the receptor-dependent translocation of components to the plasma membrane where they gain access to otherwise unavailable upstream pathway activators. Evidence suggests that both mechanisms play a role in arrestin signaling.

ERK1/2 and, to a lesser extent, c-Raf1 bind with high affinity only to the receptor-bound conformation of arrestin.[38] Although MEK1 binds equivalently to both receptor-bound and free arrestin, assembly of a productive c-Raf1–MEK1–ERK1/2 scaffolding complex requires an active GPCR docking site. Membrane translocation may also play a role. Expression of a G protein-uncoupled neurokinin NK1-arrestin2 chimera leads to constitutive activation of a pool of ERK1/2 that remains bound, along with c-Raf1 and MEK1/2 to the endosomal membrane-delimited receptor–arrestin chimera.[39] Since membrane targeting of c-Raf1 is itself sufficient to activate ERK1/2,[40] one possibility is that the arrestin functions simply to move cytosolic c-Raf1 to the membrane for activation.

The finding that plasma membrane recruitment of arrestin3 independent of receptor binding is sufficient to activate ERK1/2, albeit inefficiently, is consistent with this model.[41] ERK1/2 bound to the signalsome complex is also relatively protected from dephosphorylation by MAP kinase phosphatases, suggesting that a slower rate of inactivation also promotes sustained activity.[39] The converse situation, where receptor binding releases an arrestin-bound effector allowing it to become active, occurs during arrestin-dependent regulation of the small G protein, Ral, by Ral-GDP dissociation simulator (Ral-GDS). Ral-GDS constitutively interacts with cytoplasmic arrestin2 and 3. Upon activation of the formyl-Met-Leu-Phe receptor and arrestin recruitment, Ral-GDS is released from the arrestin complex, whereupon it regulates cytoskeleton rearrangement and exocytic granule release in polymorphonuclear neutrophilic leukocytes.[20]

In contrast to ERK1/2, both the receptor-bound and free conformations of arrestin3 are able to bind and activate the ASK1–MKK4–JNK3 cascade equivalently, indicating that the scaffold complex is preformed in cells and an activated GPCR is not required for signaling.[37] In fact all four mammalian arrestins bind JNK3 comparably and can remove it from the nucleus, where it otherwise spontaneously resides.[42] Yet only arrestin3 is able to support JNK3 activation in cells.[30,43,44] Targeted mutagenesis studies identified several residues in arrestin3, most notably Val343, that enable it alone to activate JNK3.[43] The finding that the ability of arrestins to bind pathway components is not synonymous with the ability to activate them has significant implications for the ability of arrestin scaffolds to positively or negatively regulate kinase activity in cells.

2.3. Spatial and temporal control of kinase activity

Because arrestin-dependent ERK1/2 activation occurs within relatively stable receptor–arrestin signalsomes, the arrestin-bound pool of activated ERK1/2 remains with the receptor, first appearing at the plasma membrane and later within the endosomal compartment, but not transiting to the nucleus.[14,15,45] The kinetics of pathway activation differ markedly as well. Whereas G protein activation leads to a rapid and transient rise in ERK1/2 activity, arrestin-dependent ERK1/2 activation is slow and sustained, likely because ERK1/2 activated via the arrestin pathway is protected from rapid dephosphorylation by nuclear MAPK phosphatases[34,37] (Fig. 5.2B).

These differences have profound effects on ERK1/2 function. Whereas ERK1/2 activated by classical receptor tyrosine kinase growth factor

receptors or G protein-dependent GPCR signaling is able to translocate to the nucleus and elicit a transcriptional response, ERK1/2 activated through the arrestin pathway is confined to the cytosol and silent in Elk-1 reporter assays.[25,46] Arrestin-bound ERK1/2 performs other functions, for example, regulating arrestin–clathrin interaction during GPCR endocytosis[47,48] and localized actin cytoskeletal reorganization during chemotaxis.[49] Similarly, arrestin-bound ERK1/2 mediates AngII-stimulated phosphorylation of the cytosolic targets, Mnk1 and eIF4E, leading to increased rates of mRNA translation.[35] Thus, by compartmentalizing signaling, arrestin scaffolding can change the functional consequences of pathway activation, even when the pathway is subject to convergent regulation by multiple mechanisms.

2.4. The arrestin-regulated kinome

Before considering the multitude of individual kinase and phosphatase pathways regulated by arrestins, it is worth examining the scope of arrestin-dependent effects on protein phosphorylation globally. Taking advantage of the ability of the biased angiotensin AT_{1A} receptor agonist, [Sar^1,Ile^4, Ile^8]-AngII (SII), to promote arrestin recruitment and arrestin-dependent signaling independent of significant G protein activation,[32,50] two studies have surveyed the arrestin-dependent "kinome" using whole-cell quantitative phosphoproteomic approaches. These studies point to the existence of a robust arrestin-dependent signaling network with far-reaching regulatory functions.

In AT_{1A} receptor-expressing HEK293 cells, Xiao *et al.*[51] identified 171 unique proteins whose phosphorylation increased, and 53 whose phosphorylation decreased, upon stimulation with SII, including 38 protein kinases and 3 phosphatases. A subsequent bioinformatic network analysis based on these results suggested that much of the arrestin-dependent signaling network was focused on regulation of cytoskeletal rearrangement. Using a similar strategy, Christensen *et al.*[52] performed a side-by-side global phosphoproteomic comparison of angiotensin II and SII, thus revealing the extent to which arrestin-dependent kinase regulation contributes to the overall response. These investigators detected over 1183 regulated protein phosphorylation sites out of 10,000 sites surveyed using high-resolution LTQ-Orbitrap mass spectrometry. Of these, 756 (64%) were unique to angiotensin II, 369 (34%) were regulated by both angiotensin II and SII, and 58 (5%) were unique to SII. Analysis of consensus phosphorylation sites indicated a striking difference between the kinases regulated by

G protein-dependent and arrestin-dependent signals, supporting the concept that these two networks regulate largely distinct subsets of downstream effectors. The arrestin-dependent kinase network included several kinases previously reported to be regulated by G protein-independent mechanisms, including A-Raf, Rsk-1, p70S6 kinase, and calmodulin-dependent protein kinase 2, and also pointed to protein kinase D as a downstream target of arrestin signaling.

3. POSITIVE AND NEGATIVE REGULATION OF KINASE PATHWAYS

The duality of arrestin function that characterizes its actions with respect to G protein-mediated and arrestin-mediated signaling processes has curious parallels in its kinase and phosphatase scaffolding role. The binding of pathway components to arrestins not only facilitates ligand-dependent arrestin signaling but also allows arrestins to sequester kinases and regulatory proteins in a manner that dampens basal pathway activity.

3.1. Negative regulation of second messenger-dependent protein kinases

The simplest negative regulatory role of arrestin scaffolds is one that complements its roles in GPCR desensitization and sequestration. Two enzymes involved in second messenger breakdown have been reported to bind arrestins: type 4D cAMP phosphodiesterases (PDE4D) and DGK. Arrestin-dependent recruitment of these enzymes to the locus of second messenger production serves as an additional means of limiting the magnitude and duration of G protein-mediated activation of the second messenger-dependent protein kinases, protein kinase (PK)A and PKC. Arrestins 2 and 3 interact with all five PDE4D isoforms, PDE4D1–5.[17] The Gs-coupled β2-adrenergic receptor forms a signaling complex with arrestin3 and PDE4D3 and PDE4D5, leading to accelerated cAMP degradation. Recruitment of PDE4D into the signalsome appears to be highly receptor specific, since the closely related β1-adrenergic receptor was shown to recruit a different alternative-spliced isoform, PDE4D8, and to do so without the aid of arrestin.[53] Arrestin-dependent recruitment of DGK appears to dampen M1 muscarinic receptor-mediated PKC activity.[18] DGK converts diacylglycerol produced by PLCβ to phosphatidic acid. Besides terminating PKC activity, this mechanism may generate

physiologically relevant concentrations of phosphatidic acid, a precursor of the multifunctional lipid second messenger, lysophosphatidic acid (LPA).

3.2. Positive and negative regulation of arrestin-associated kinases

The complexity of arrestin-dependent regulation of kinase activity can be illustrated by two examples: its opposing effects on ERK1/2 and NF-κB signaling and its bidirectional regulation of the PP2A–AKT–GSK3 cascade. As previously discussed, ERK1/2 is recruited preferentially to the receptor-bound conformation of arrestin2 and 3,[38] permitting GPCRs to selectively activate ERK1/2 within receptor–arrestin signalsomes.[14,15] The resulting spatial compartmentalization of ERK1/2 leads to increased phosphorylation of cytosolic targets,[35,47–49] while inhibiting its ability to stimulate Elk-1-dependent transcription.[25,46] At the same, the dominant effect of arrestins on nuclear factor (NF)-κB signaling is to dampen pathway activity. In NF-κB signaling, phosphorylation of the inhibitory protein, IκBα, by IκBα kinases accelerates its proteosomal degradation, permitting nuclear translocation of NF-κB and increased NF-κB transcriptional activity. IκBα binds arrestin3, while both arrestin2 and 3 interact with IκB kinase α/β and NR-IκB-inducing kinase.[54,55] Downregulating arrestin2 expression increases NF-κB activation by tumor necrosis factor α (TNFα), consistent with the hypothesis that arrestins tonically inhibit NF-κB signaling by protecting IκBα from degradation.[19] In HEK293 cells, downregulating arrestin expression attenuates toll-like receptor (TLR)4-mediated ERK1/2 activation while simultaneously enhancing NF-κB reporter activity, suggesting that arrestins exert opposing effects on the ERK1/2 and NF-κB pathways.[56]

The situation with respect to PP2A–AKT–GSK3 signaling is similarly complex. β-Catenin and Akt signaling is regulated by an arrestin3 signalsome complex composed of the catalytic subunit of PP2A, Akt, and glycogen synthase kinase 3β (GSK3β).[23] As with NF-κB, the dominant arrestin effect is to dampen β-catenin signaling. Within the arrestin3 complex, PP2A maintains Akt in an inactive state by dephosphorylating Thr^{308}. Since Akt phosphorylation of GSK3β inhibits its activity, keeping Akt inactive increases GSK3α/β activity. GSK3β, in turn, phosphorylates β-catenin, accelerating its degradation and inhibiting β-catenin-dependent transcription. Predictably, brain extracts from arrestin3 null mice show higher levels of β-catenin expression, presumably reflecting the loss of tonic arrestin-mediated Akt inhibition.[57] On the other hand, a phosphoproteomic screen

of SII-mediated protein phosphorylation identified two peptide inhibitors of PP2A (I1PP2A and I2PP2A) as targets for increased phosphorylation.[58] Agonist-stimulated phosphorylation of I2PP2A was associated with rapid and transient inhibition of the arrestin3-associated pool of PP2A, leading to activation of Akt and increased phosphorylation of GSK3β in an arrestin signalsome complex. Thus, while arrestins maintain tonic inhibition of GSK3β under homeostatic conditions *in vivo*,[57] mechanisms may exist to release that inhibition in response to GPCR stimulation.

4. ARRESTIN-REGULATED KINASE AND PHOSPHATASE PATHWAYS

Table 5.1 summarizes many of the experimentally validated arrestin-dependent GPCR-signaling pathways reported to date.[10,72] The evidence supporting their existence ranges from coprecipitation studies using over-expressed pathway components to isolation of endogenous arrestin–effector complexes from native tissues and from loss of function studies using arrestin dominant-negative mutants and isoform-selective RNA interference to rescue studies performed using arrestin2/3 null murine embryo fibroblasts (MEFs). Viewed as a whole, arrestin-regulated kinase and phosphatase signaling appear to encompass a fairly discrete set of functions, linking GPCRs to receptor and nonreceptor tyrosine kinases, MAPKs, regulators of NF-κB and β-catenin signaling, and a few protein phosphatases and lipid kinases. Many of these putative effectors are not known to be regulated by heterotrimeric G protein subunits, suggesting that these GPCR–arrestin–effector pathways function in parallel with GPCR–G protein–effector pathways to add additional dimensions to GPCR signaling.

4.1. Tyrosine protein kinases

4.1.1 Src family nonreceptor tyrosine kinases

Arrestins bind to several members of the Src family of nonreceptor tyrosine kinases and recruit them to activated GPCRs. Arrestin-dependent recruitment of c-Src to β2-adrenergic receptors on the plasma membrane can be visualized after isoproterenol stimulation.[6] As with several other nonreceptor arrestin-binding partners, Src kinases appear to make contact with arrestins at several points. The N-terminus of arrestin2 is proline rich and contains three PXXP motifs that interact with the Src homology (SH)3 domain of c-Src.[6] Additional contacts involving the c-Src SH1 (catalytic) domain confer added binding affinity.[59] In contrast, arrestin1 has only a

Table 5.1 Arrestin-regulated kinase and phosphatase pathways

Effector	Arrestin	Reported functions	References
Tyrosine protein kinases			
Src family tyrosine kinases c-Src, c-Yes, c-Hck, c-Fgr, c-Fyn	Arrestin1 Arrestin2 Arrestin3	ERK1/2 activation Dynamin 1 phosphorylation Exocytosis/granule release Phosphorylation/destabilization of GRK2 FAK phosphorylation EGF receptor transactivation Phosphorylation of β2-adaptin subunit of AP-2 complex	6,11 59 12 60 61 62 48
SER/THR protein kinases			
c-Raf1–MEK1/2–ERK1/2	Arrestin2 Arrestin3	Activation of cytosolic ERK1/2 p90RSK phosphorylation Actin cytoskeletal reorganization/chemotaxis ERK1/2-dependent transcription Mnk1/eIF4E phosphorylation/protein translation	14,15 63 49 64 35
ASK1–MKK4–JNK3	Arrestin1 Arrestin2 Arrestin3	Inhibition of cytosolic JNK3 Activation of cytosolic JNK3	36 13
ASK1–MKK3–p38 MAPK	Arrestin2 Arrestin3	Activation of p38 MAPK Inhibition of p38 MAPK	65,66 67
IκBα–IκB kinase α/β	Arrestin2 Arrestin3	Attenuation of NF-κB signaling	19,55,56
Casein kinase 2	Arrestin3	Activation of CK2	58
Protein phosphatases			
PP2A–Akt–GSK3β	Arrestin3	Activation of Akt Inactivation of Akt-GSK3β/Activation of β-catenin signaling	58,68 23
Cofilin, chronophin, LIM kinase	Arrestin3	Actin cytoskeletal reorganization/chemotaxis	21
SHP-1, SHP-2	Arrestin3	Inhibition of NK cell cytotoxicity	69
Lipid kinases			
Diacylglycerol kinase	Arrestin2 Arrestin3	Attenuation of PKC signaling	18
Phosphatidylinositol 3-kinase	Arrestin2	Inhibition of PI3K	70
PI 4-phosphate 5-kinase Iα	Arrestin2 Arrestin3	Control of GPCR internalization	71

single PXXP motif and binding appears to involve the c-Src SH2 domain.[73] It is unclear whether c-Src exhibits a binding preference for the receptor-bound conformation of arrestins, but immunostaining for the active (Y^{530} dephosphorylated) form of c-Src suggests that c-Src in the receptor–arrestin complex is active.[6] It is also unclear whether the association of arrestin and c-Src is regulated, although phosphorylation of Ser^{412} in the C-terminus of arrestin2, which destabilizes the receptor–arrestin complex, has been reported to disrupt the arrestin–Src interaction.[47]

Arrestin-dependent recruitment of Src family kinases appears to regulate clathrin-dependent GPCR endocytosis. Arrestin-scaffolded c-Src phosphorylates GRK2, providing negative feedback on receptor desensitization by destabilizing GRK2 and promoting its entry into the proteosome pathway where it is rapidly degraded.[60] Arrestin–Src binding is required for phosphorylation of Tyr^{497} of dynamin 1, which regulates dynamin self-assembly.[59,74,75] Expression of a Y497F mutant of dynamin 1 impairs the internalization of both the β2-adrenergic[74] and the M2 muscarinic acetylcholine receptor.[76] The β2-adaptin subunit of AP-2 is another endocytic protein whose regulation by Src is arrestin dependent.[48,77,78] c-Src stabilizes the constitutive association between arrestin3 and β2-adaptin independent of its kinase activity. Src-mediated phosphorylation of β2-adaptin Tyr^{737} occurs in clathrin-coated pits in response to AT_{1A}, β2 adrenergic, V2 vasopressin, or B2 bradykinin receptor activation, leading to dissociation of AP-2 from the complex. If β2-adaptin phosphorylation is blocked, receptor–arrestin complexes are retained at the membrane.

The involvement of arrestin–Src complexes in vesicle trafficking extends to exocytosis. Arrestin-dependent activation of the Src family kinases, c-Hck and c-Fgr, by the interleukin 8 receptor (CXCR1) appears to be important for granule release, since expression of P91G/P121G arrestin2 with mutations in the PXXP motifs antagonizes CXCR1-induced exocytosis in granulocyte cells.[12] Similarly, the endothelin type A receptor assembles an arrestin2-dependent complex with c-Yes that positively regulates endothelin-1-stimulated translocation of the glucose transporter Glut4 to the plasma membrane.[79]

Arrestin-dependent Src signaling has been implicated in several other GPCR-regulated processes, notably activation of ERK1/2. Src recruitment to β2-adrenergic receptors contributes to ERK1/2 MAP activation.[6] The neurokinin NK1 receptor forms a receptor–arrestin–c-Src signalsome in response to substance P stimulation.[11] Assembly of this signalsome has been implicated in NK1 receptor-mediated ERK1/2 activation, cell survival, and

proliferation. D2 dopamine receptor activation of the NF-κB pathway reportedly requires c-Src as well as Gαi proteins, and this response is potentiated by overexpression of arrestin2.[80] c-Src is also recruited to rhodopsin–arrestin1 complexes, which concentrate in the rod outer segment compartment upon light exposure.[73] It has been proposed that c-Src binding to arrestin1 promotes the formation of high-affinity phospho-Tyr binding sites in the rod outer segment that lead to translocation of SH2-containing proteins.

4.1.2 Receptor tyrosine kinases

Members of EGF receptor family of receptor tyrosine kinases (HER1–4) are key convergence points for mitogenic stimuli. A well-characterized mechanism whereby GPCRs affect cell proliferation and survival is by stimulating the matrix metalloprotease (MMP)-dependent shedding of preformed EGF-family growth factors, leading to paracrine transactivation of EGF receptors (reviewed in Ref. 81). In some settings, arrestin–Src complexes play a role in GPCR-mediated EGF receptor transactivation, and arrestin-dependent activation of the ERK1/2 cascade downstream of transactivated EGF receptors can stimulate transcription and promote cell proliferation. This contrasts with direct arrestin-dependent scaffolding mechanism of ERK1/2 activation, wherein the signalsome-bound active ERK1/2 is usually transcriptionally repressed.[25,46]

The luteinizing hormone (LH) receptor activates c-Fyn in an arrestin3-dependent manner.[61] Downregulating arrestin expression reduces the rate of internalization of hCG by 50% and inhibits LH receptor-mediated activation of c-Fyn, phosphorylation of the antiapoptotic focal adhesion kinase (FAK), and the release of EGF-like growth factors. In HEK293 cells expressing β1-adrenergic receptors, EGF receptor transactivation and ERK1/2 activation are inhibited by downregulation of arrestin2 or 3, or GRK5 or 6, inhibiting Src or MMP activity, or exposure to a heparin-binding-EGF neutralizing antibody, suggesting that β1-receptor-mediated EGF receptor transactivation is arrestin dependent.[62] Consistent with this, a mutant β1-receptor lacking 14 GRK phosphorylation sites in its C-terminal tail (-GRKβ1), which cannot undergo arrestin-dependent desensitization, fails to transactivate EGF receptors despite exaggerated G protein activation. Transgenic mice expressing the -GRKβ1 receptor in cardiomyocytes develop more severe dilated cardiomyopathy in response to chronic isoproterenol stimulation, with significantly increased LV end-diastolic dimension, decreased fractional shortening, and increased

myocardial apoptosis. In this model, inhibiting EGF receptors worsens the dilated cardiomyopathy, suggesting a protective role for transactivated EGF receptors in the heart.[62]

Arrestin signaling is not the only mechanism of GPCR-stimulated ectodomain shedding, and, in some cases, arrestin-mediated GPCR desensitization antagonizes signaling by transactivated EGF receptors. In arrestin2/3 null MEFs, ERK1/2 activation by LPA receptors reflects primarily G protein-dependent transactivation of EGF receptors.[64] Because LPA receptor desensitization is impaired, the EGF receptor-dependent ERK1/2 signal is persistent, lasting for several hours in the continued presence of LPA. Reintroducing arrestin3, which restores desensitization, makes the transactivation-dependent signal transient, such that it contributes significantly to ERK1/2 activation only during the first few minutes of stimulation. At the same time, arrestin3 confers a long-lasting EGF receptor-independent ERK1/2 signal that presumably reflects activation of the arrestin-scaffolding pathway. Whereas most of the early LPA-stimulated transcriptional responses in arrestin2/3 null MEFs are EGF receptor-dependent, expression of arrestin3 attenuates EGF receptor-dependent transcription and enables LPA to elicit EGF receptor-independent transcription.

4.2. Mitogen-activated protein kinases

4.2.1 Extracellular signal-regulated kinases

ERK1/2 activity is required for G0–G1 cell-cycle transition and the passage of cells through mitosis or meiosis.[82] Heptahelical receptors employ multiple mechanisms to activate ERK1/2, often simultaneously, from PKA- and PKC-dependent signals downstream of heterotrimeric G proteins, to transactivation of EGF receptors, to signals transmitted via arrestins.[81,83] As a result, GPCR-mediated ERK1/2 activation in any given setting is complex, as are the functional consequences. Regarding arrestin-dependent ERK1/2 activation, several factors influence the spatial distribution and duration of ERK1/2 activity including the specific GRK and arrestin isoforms acting on the receptor, and the stability of the receptor–arrestin interaction.

Although arrestins 1, 2, and 3, but not arrestin4, are able to bind ERK1/2,[84] evidence suggests that, at least with some receptors, the arrestin2 and 3 isoforms play opposing roles in arrestin-mediated signaling and desensitization. In HEK293 cells expressing AT_{1A} receptors, isoform-selective down-regulation of endogenous arrestin3 reduces angiotensin II-stimulated ERK1/2 activation by about 50% and abrogates activation the arrestin pathway-selective ligand SII. Paradoxically, silencing arrestin2 expression

has the opposite effect, enhancing the arrestin-dependent component of the ERK1/2 signal,[34,46] suggesting that, with respect to ERK1/2 activation, arrestin3 is the signaling arrestin isoform, while arrestin2 functions only in desensitization. Arrestin3 is certainly the key isoform for Class A receptors like the β2 adrenergic, which do not bind arrestin2 efficiently,[85] but the dichotomy of isoform-specific arrestin function does not appear to hold for all Class B receptors. Arrestin-dependent ERK1/2 activation by the type 1 parathyroid hormone receptor, for example, is inhibited when either isoform is downregulated, suggesting that both are required to assemble functional signalsomes.[86]

Additional evidence suggests that arrestins adopt different "active" conformations depending on which GPCR they bind and which GRK phosphorylated the receptor. Evidence of the former comes from characterization of arrestin ubiquitination. Ubiquitination of lysines 11 and 12 of arrestin3 is necessary for it to remain stably bound to the AT_{1A} receptor, yet an arrestin3 (K11,12R) mutant is still ubiquitinated and fully functional when recruited to the vasopressin V2 receptor.[45] All 31 lysines must be mutated before arrestin3 ubiquitination is lost upon β2-adrenergic receptor binding.[87] The variability suggests that either the conformation or the accessibility of surface epitopes on arrestin3 differs depending on the GPCR-binding partner. Additional support for different arrestin conformations comes from data obtained using isoform-selective silencing of GRKs that suggest GRK2 and GRK3 phosphorylation of the AT_{1A} and V2 receptors promotes arrestin-dependent desensitization, while GRK5 and GRK6 appear to be exclusively responsible for initiating arrestin-dependent ERK1/2 activation.[88,89] Similar work with the β2-adrenergic receptor suggests that GRK2 and GRK6 phosphorylate the receptor C-terminus at different sites, and that only the GRK6-induced pattern of phosphorylation supports arrestin-dependent ERK1/2 activation.[90] This has led to the hypothesis that different GRKs establish a phosphorylation "barcode" that imparts distinct arrestin3 conformations to regulate its functional activity.

Signalsomes regulate the spatial, temporal, and functional characteristics of ERK1/2. When recruited to a Class B receptor, active ERK1/2 accumulates in early endosomes, failing to translocate to the cell nucleus. Wild-type PAR2 receptors predominantly utilize the arrestin-dependent pathway to activate ERK1/2.[14] As a result, ERK1/2 is excluded from the nucleus and does not stimulate proliferation. In contrast, a C-terminal phosphorylation site mutant PAR2, that does not bind arrestins or internalize, activates ERK1/2 via a G protein-dependent pathway that promotes its nuclear

translocation and stimulates proliferation. Similarly, the wild-type AT_{1A} receptor activates ERK1/2 using both G protein- and arrestin-dependent pathways, increasing both cytosolic and nuclear ERK1/2,[25,34] whereas a G protein-uncoupled DRY–AAY AT_{1A} receptor mutant, which utilizes only the arrestin pathway, only activates cytosolic ERK1/2 and fails to elicit a detectable transcriptional response.[46] Native V2 receptors similarly engage both pathways. Exchanging the V2 receptor C-terminus for that of the Class A β2-adrenergic receptor, which converts the receptor from stable to transient arrestin binding, increases the proportion of ERK1/2 that enters the cell nucleus and permits the chimeric receptor to stimulate cell proliferation. The opposite effect is obtained when the V2 receptor tail is appended to the β2 receptor.[31]

Whether ERK1/2 activated by Class A receptors using the arrestin pathway is transcriptionally competent is less clear. Class A receptors, like the β2-adrenergic and LPA receptors, also use arrestin scaffolds to activate ERK1/2, but the transient nature of the receptor–arrestin interaction does not support endosomal targeting.[64,85] Reintroduction of arrestin3 into arrestin2/3 double null MEFs restores arrestin-dependent ERK1/2 activation, and while it represses transcription mediated through G protein-dependent transactivation of EGF receptors, it enables LPA to elicit ERK1/2 dependent, but EGF receptor-independent transcription.[64] Such findings suggest that dissociation of the LPA receptor–arrestin complex upon internalization may permit ERK1/2 activated by the arrestin pathway to enter the cell nucleus.

The spatial constraint imposed by the assembly of stable signalsomes appears to direct ERK1/2 kinase activity toward membrane or cytosolic substrates. ERK1/2 phosphorylates Ser^{412} in the C-terminus of arrestin2, limiting its ability to bind clathrin.[47] Arrestin2 in the cytosol is almost stoichiometrically phosphorylated on Ser^{412}, and dephosphorylation of Ser^{412} upon receptor binding promotes receptor internalization and ERK1/2 activation. Rephosphorylation by ERK1/2 in the signalsome complex probably provides either negative feedback regulation of receptor endocytosis or facilitates receptor internalization by promoting dissociation of arrestin and clathrin, allowing the receptor to exit clathrin-coated vesicles. Arrestin-dependent targeting of ERK1/2 to the plasma membrane also appears to play a role in chemotaxis. During PAR2-induced chemotaxis, PAR2 receptor–arrestin–ERK1/2 complexes localize to the leading edge of the cell where ERK1/2 activity is required for actin cytoskeletal reorganization.[49] Other cytosolic ERK1/2 substrates include the ribosomal S6 kinase,

p90RSK. ERK1/2 phosphorylation of p90RSK is activated by a mutant AT_{1A} receptor with a deletion in its second intracellular loop that inhibits G protein coupling.[63] This arrestin-dependent activation of the ERK1/2 substrate p90RSK acts in concert with another arrestin-mediated signal, phosphatidylinositol 3-kinase (PI3K)–AKT to downregulate phospho-BAD, inducing antiapoptotic cytoprotective effects in rat vascular smooth muscle.[91] Using RNA interference to downregulate arrestin3, it has also been possible to show that arrestin-dependent ERK1/2 activation by the AT_{1A} receptor mediates phosphorylation of Mnk1 and eIF4E, increasing rates of mRNA translation.[35]

4.2.2 c-Jun N-terminal kinase 3

JNK1–3 are stress-activated kinases that regulate apoptosis by stimulating cytochrome *C* release from the mitochondria during cellular stress and control transcription by phosphorylating the transcription factor c-Jun.[92] There are three JNK isoforms, of which JNK1/2 are widely expressed, while JNK3 is highly expressed only in brain, heart, and testes.[93] JNK2 and JNK3 were originally found to interact with arrestin3 in yeast two-hybrid screens, but only JNK3 interacts with arrestins in mammalian cells.[13] The three components of the JNK3 cascade, Ask1, MKK4, and JNK3, are able to bind all four arrestin isoforms. While all four arrestins can cause JNK3 to redistribute from its normal nuclear location into the cytosol, only arrestin3 is able to potentiate JNK3 activation.[36,42]

It is unclear whether GPCRs exert any control over arrestin3-dependent activation of JNK3 or indeed whether activation of arrestin3–JNK3 has a physiologic role.[94] Interestingly, JNK3 exhibits higher affinity for the "inactive" conformation of arrestins, suggesting that its dominant role is to repress basal JNK3 signaling.[36,42] Even though the JNK3 bound to arrestin3 is active, it is sequestered in the cytosol away from its nuclear transcription factor targets. The original study, performed in transfected cells, reported that stimulation of AT_{1A} receptors activated JNK3 and caused it to colocalize with arrestin3 in endosomal vesicles,[13] but later work performed with the β2-adrenergic receptor found no evidence of receptor-mediated JNK3 activation under conditions where ERK1/2 was being robustly activated via the arrestin3 pathway. To the contrary, expression of inactive arrestin3 mutants that do not bind GPCRs increased basal JNK3 phosphorylation while simultaneously decreasing receptor-catalyzed ERK1/2 activation, confirming that while arrestin3-dependent ERK1/2 activation is receptor dependent, JNK3 activation is not.[30] In a more

physiologic animal model of cerebral ischemia, the angiotensin receptor blocker, losartan, has been reported to attenuate neuronal damage by inhibiting the assembly of an AT_{1A} receptor–arrestin3–Ask1–MKK4-signaling module and repressing the activation of JNK3, c-jun, and caspase-3, and the release of cytochrome *C*.[95]

4.2.3 p38 MAP kinase

Although the molecular mechanisms of activation have not been characterized in any detail, several studies have reported arrestin-dependent regulation of the p38 MAP kinase cascade. In HeLa and HEK293 cells, overexpression of arrestin3 enhanced, and downregulation inhibited, activation of both p38 MAPK and ERK1/2 following stimulation of the chemokine receptor CXCR4 by its ligand, stromal cell-derived factor 1α.[65] Inhibiting p38 MAPK, but not ERK1/2, blocked arrestin3-dependent chemotaxis, suggesting a specific role for arrestin-dependent p38 MAPK in CXCR4 signaling. In primary cultured astrocytes, κ-opioid receptor-stimulated activation of p38 MAPK appears to involve GRK3 and arrestin3.[66] p38 activation does not occur in astrocytes derived from κ-opioid receptor or GRK3 null mice, or following downregulation of arrestin3 expression.

As with ERK1/2, however, the dominant effect of arrestins in other systems appears to attenuate p38 MAPK activation via G protein-dependent pathways. Arrestin2/3 null MEFs exhibit greatly enhanced activation of ERK1/2, JNK1/2, and p38 MAPK in response to the CXCR2 agonist, interleukin 8.[67] Activation of the stress kinases, JNK1/2 and p38 MAPK, was dependent on reactive oxygen species generated by NADPH oxidase, and arrestin expression conferred protection from oxidative burst-induced cell death resulting from sustained CXCR2 activation. Similarly, β2-adrenergic receptor-mediated regulation of immunoglobulin IgE expression on CD40L/interleukin-4-activated B lymphocytes involves PKA and p38 MAPK, but not arrestins. β2-Adrenergic receptor stimulation leads to Gs/cAMP/PKA-dependent phosphorylation and inactivation of the hematopoietic protein tyrosine phosphatase, HePTP, which releases bound p38 MAPK, making more available for phosphorylation and subsequent IgE regulation.[96] In this setting, arrestin-dependent β2-adrenergic receptor desensitization would attenuate, not enhance p38 MAPK activity.

Besides CXCR4-mediated chemotaxis,[65] arrestin-dependent regulation of p38 MAPK has been implicated in several cellular processes. In polymorphonuclear neutrophils, clathrin-mediated endocytosis of the platelet-activating receptor (PAF) requires p38 MAPK-dependent

rearrangement of actin bundles. Activation of PAF receptors leads to recruitment and activation of an arrestin2–ASK1–MKK3–p38 MAPK signalsome, which is required for subsequent cell polarization, actin bundle formation, and receptor endocytosis.[97] While the antiapoptotic effects of arrestins are mediated primarily through activation of the prosurvival kinase AKT (*vide infra*), such that genetic deletion of either arrestin increases apoptosis after serum withdrawal, deletion of both arrestin2 and 3 is paradoxically protective.[98] Individual deletion of either arrestin2 or 3 enhances starvation-induced activation of ERK1/2 and p38 MAPK and caspase 3 cleavage, while decreasing AKT activity. When both are deleted, starvation-induced ERK1/2 and p38 MAPK activation returns to wild-type levels. This suggests that both arrestins are required to maintain a balance between proapoptotic p38 MAPK and antiapoptotic AKT-signaling pathways.

4.3. Other SER/THR kinases

4.3.1 Regulators of nuclear factor-κB

As previously discussed, arrestins2 and 3 appear to act as negative regulators of NF-κB transcription by sequestering the NF-κB inhibitor, IκBα, and protecting it from phosphorylation by IκBα kinases, which promotes its degradation. β2-Adrenergic receptor stimulation increases arrestin3 binding to IκBα, preventing its phosphorylation-dependent degradation and inhibiting IL-8-stimulated NF-κB activity.[54,55] Consistent with this, TLR4-mediated activation of an NF-κB reporter is enhanced by downregulating arrestin expression.[56] Arrestin binding to the E3 ubiquitin ligase, TRAF6, probably augments the inhibition of TLR signaling.[99] TRAF6 is normally recruited to TLR/IL-1 family receptors where it facilitates IκB kinase and NF-κB activation. Binding of TRAF6 to arrestin2 and 3 in response to lipopolysaccharide or IL-1 stimulation prevents TRAF6 oligomerization and autoubiquitination, negatively regulating LPS and IL-1 signaling.

The capacity to negatively regulate NF-κB transcriptional pathways involved in cytokine signaling suggests that arrestins may dampen immune responses by inhibiting the production of proinflammatory cytokines. Consistent with this, isolated peritoneal neutrophils from arrestin3 null animals exhibit increased basal and LPS-stimulated TNFα and IL-6 production.[100] Arrestin effects in cancer may also derive in part from modulation of NF-κB signaling. Loss of expression of the type III transforming growth factor-β (TβRIII) occurs in a variety of human malignancies. TβRIII is thought to function as a tumor suppressor by reducing cell motility. Although not a GPCR, clathrin-dependent endocytosis of TβRIII and downregulation

of TGFβ signaling depends on the interaction with arrestin3.[101] *In vitro* evidence suggests that arrestin3 plays a key role in the ability of TβRIII to inhibit cell migration. In breast and ovarian cancer cell lines, activation of the small GTPase Cdc42 by TβRIII alters actin cytoskeletal rearrangement and reduces random cell migration.[102] A TβRIII mutant unable to interact with arrestins fails to inhibit migration and the wild-type receptor effect is blocked by downregulating arrestin3. In addition, the interaction between TβRIII and arrestin3 negatively regulates NF-κB transcriptional activity, further inhibiting cell migration.[103]

4.3.2 Casein kinase 2

Casein kinase 2 (CK2) is a ubiquitously expressed, constitutively active Ser/Thr protein kinase that performs diverse functions related to cell survival and tumorigenesis. The catalytic subunit of CK2 was identified in a proteomic screen of arrestin3 binding proteins as well as a phosphoproteomic screen of AT_{1A} receptor-mediated phosphorylation following stimulation with the arrestin-selective biased agonist, SII.[51,58] Since CK2 has been implicated in phosphorylation of Thr^{383} of arrestin3, which destabilizes the interaction between arrestin3 and β2-adrenergic receptors,[104] it is possible that arrestin recruitment of CK2 plays a role similar to that proposed for ERK1/2-mediated phosphorylation of arrestin2 Ser^{412}.

4.4. Protein phosphatases

4.4.1 PP2A–AKT–GSK3β

The Ser/Thr phosphatase PP2A is ubiquitously expressed and has broad substrate specificity. The PP2A holoenzyme is heterotrimeric, composed of regulatory A and B subunits that target the catalytic C subunit to specific intracellular locations. The PP2A catalytic subunit was identified as an arrestin3-interacting protein in a proteomic screen,[51] and a native arrestin3–PP2A–AKT–GSK3β complex has been purified from the D2 dopamine receptor-rich striatum of mice.[23] The regulation of PP2A–AKT–GSK3 signaling by arrestins is probably one of the most physiologically important arrestin-regulated signaling processes, being critically involved in both AKT-dependent survival signaling and regulation of β-catenin-mediated transcription. As previously discussed, the major homeostatic function of the complex in the striatum is to modulate D2 dopamine receptor-mediated behaviors by tonic repression of β-catenin signaling.[23,57] At the same time, AT_{1A} receptor-mediated, G protein-independent, phosphorylation of the PP2A inhibitor, I2PP2A, may provide a mechanism for acute modulation

of AKT and GSK3β kinase activity by transiently inhibiting PP2A in the signalsome complex.[58] PAR1 receptor activation also reportedly causes rapid activation of AKT through an unknown arrestin2-dependent mechanism.[68]

Dopaminergic neurotransmission in the brain regulates behavioral responses such as locomotor activity and neural reward mechanisms. Loss of dopaminergic cells in the substantia nigra leads to a loss of locomotor control in Parkinson's disease. Conversely, D2 dopamine receptor antagonists are effective neuroleptic drugs used in the treatment of schizophrenia and attention-deficit hyperactivity disorder, which are thought to result from excess dopaminergic neurotransmission. Several lines of evidence suggest that arrestin-signaling complexes regulate dopamine-dependent behaviors. Locomotor hyperactivity induced by the dopaminergic drug apomorphine, a D2 receptor agonist, is reduced in arrestin3 knockout mice.[57] Similarly, the hyperactivity displayed by dopamine-transporter knockout mice, which results from increased synaptic dopamine concentration, is reduced when DAT knockout mice are crossbred with arrestin3 knockouts, a paradoxical result, since G protein-mediated responses would be enhanced by the loss of arrestin-dependent desensitization. The molecular basis of these effects may lie in the scaffolding of PP2A–AKT–GSK3 by arrestin3. Amphetamine treatment, which increases synaptic dopamine release, increases the PP2A–AKT association in wild type, but not arrestin3 knockout mice, suggesting that arrestins mediate the interaction.[57] Directly inhibiting PP2A or GSK3β also mimics the effect of arrestin3 knockout on hyperactive locomotor activity in DAT knockout mice, suggesting that dopamine-mediated activation of GSK3β results from PP2A-dependent AKT inhibition that is scaffolded by arrestin3.[23]

Arrestin3–PP2A–AKT scaffolds may underlie the mechanism of action of many antipsychotic drugs. Lithium, a mood stabilizer used in the treatment of schizophrenia, modulates dopamine-dependent behavior in mice such as horizontal activity. GSK3β haploinsufficient mice exhibit augmented lithium-induced antidepressant and anxiolytic effects compared to wild-type animals, suggesting that lithium acts by inhibiting GSK3β. Therapeutic concentrations of lithium disrupt the interaction between arrestin3, AKT, and PP2A, relieving PP2A-mediated negative regulation of AKT, allowing it to phosphorylate and inactivate GSK3β.[57] The arrestin3–PP2A–AKT complex requires magnesium, and lithium is thought to destabilize the complex by competing for magnesium binding. The clinical efficacy of essentially all other classes of antipsychotic drug correlates directly to their dopamine

D2-receptor binding affinity and ability to antagonize the receptor. In an *in vitro* screen using fluorescence-based reporters, it was found that while different classes of antipsychotics exhibit complex efficacy profiles with respect to D2 receptor–G protein coupling, they share the property of antagonizing the D2 receptor–arrestin3 interaction.[105]

PP2A also plays an important role in receptor trafficking and resensitization, and some of these effects may be arrestin dependent. Arrestin2-bound PP2A reportedly dephosphorylates Ser^{412} on arrestin2, a step that regulates the interaction between arrestin and the clathrin-coated pit and subsequent internalization.[106] Dephosphorylation of GRK-phosphorylated receptors, a prerequisite for receptor resensitization, also involves PP2A. A 150-kDa oligomeric form of PP2A catalyzes the dephosphorylation of β2- and α2-adrenergic receptors.[107] The acidic microenvironment of endosomes is important for PP2A association with internalized β2-adrenergic receptors, their dephosphorylation, and subsequent recycling to the plasma membrane.[108] At present, it is not clear whether the PP2A pool involved in receptor dephosphorylation is targeted by the arrestin. Finally, PP2A is known to promote ERK1/2 activation by acting on c-Raf1 Ser^{259}, an inhibitory site that must be dephosphorylated for Raf activation.[109] Since PP2A both positively regulates c-Raf[109,110] and negatively regulates ERK1/2,[111,112] its presence in the signalsome may play a role in arrestin-dependent ERK1/2 regulation.

4.4.2 Cofilin–chronophin–LIM kinase

Arrestin kinase/phosphatase scaffolds play a central role in the control of GPCR-mediated chemotaxis, the process whereby migrating cells follow a concentration gradient to its source. Chemoattractant receptor activation induces actin cytoskeletal rearrangement forming leading and trailing edges. A dominant pseudopodium forms at the leading edge that protrudes forward driven by F-actin polymerization and actin-myosin contraction forces.[113] Splenocytes derived from arrestin3 null mice exhibit strikingly impaired chemotactic responses to stromal cell-derived factor-1, CXCL12.[114] While impaired gradient sensing due to the loss of arrestin-mediated desensitization might be a contributing factor,[115] evidence suggests that arrestin-dependent regulation of ERK1/2 and cortical actin assembly at the leading edge is required for GPCR-mediated chemotaxis.[49,116–118] In addition, arrestins scaffold a complex containing the actin filament-severing protein, cofilin, LIM kinase, and the cofilin-specific phosphatase, chronophin, which is required for the dephosphorylation and activation of cophilin.[21] Arrestin-bound

cophilin generates the free barbed ends on actin filaments that permit filament extension. Arrestins also affect cell shape change by interacting with the actin-bundling protein, filamin A. Assembly of an AT_{1A} receptor–arrestin–ERK1/2–filamin A complex is required for the formation of membrane ruffles in Hep2 cells.[119]

4.4.3 Protein tyrosine phosphatases

The Src homology 2-containing protein tyrosine phosphatase, SHP-1, modulates AKT activation by the ghrelin receptor, growth hormone secretagogue receptor type 1α (GHSR1α). In adipocytes, ghrelin activates AKT by both an early pertussis toxin-sensitive Gi/o-mediated pathway and a slower arrestin-scaffolded pathway.[120] AKT activation by GHSR1α involves tyrosine phosphorylation of the PI3K regulatory subunit p85, leading to an increase in c-Src and phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylation and activation of AKT. SHP-1 is present in a receptor-associated arrestin-scaffold complex, where it attenuates ghrelin-induced c-Src and AKT activation. An arrestin3-dependent mechanism also appears to negatively regulate the activity of natural killer cells, a key component of the innate immune response. Arrestin3 mediates recruitment of SHP-1 and SHP-2 to KIR2DL1, an inhibitory receptor of natural killer cells, facilitating downstream inhibitory signaling.[69]

4.5. Lipid kinases

4.5.1 Diacylglycerol kinases

DGKs convert diacylglycerol produced by PLCβ to phosphatidic acid. As previously discussed, classical arrestin-dependent desensitization of M1 muscarinic acetylcholine receptor signaling via the Gq/11–PLCβ–PKC pathway is augmented by the arrestin-dependent recruitment of DGK to the receptor, which accelerates second messenger degradation.[18]

4.5.2 Phosphatidylinositol 3-kinase

As an upstream modulator of AKT, PI3K plays an integral role in several arrestin scaffold functions. PI3K activity is required to translocate PDK1 the plasma membrane. PDK1 is a constitutively active kinase and, upon translocation, phosphorylates AKT Thr^{308}, leading to AKT activation. As previously discussed, arrestin scaffolding of PP2A, which dephosphorylates AKT Thr^{308}, restrains AKT activity in brain.[22,23] While there is no known mechanism whereby arrestins promote PI3K activity, there are data suggesting that they inhibit it. PAR2 receptors stimulate PI3K activity

through a Gq/11–Ca^{2+}-dependent pathway involving the nonreceptor tyrosine kinase Pyk2 and c-Src.[70] Arrestin2 can directly associate with the catalytic p110α subunit of PI3K and inhibit its activity. PI3K is recruited into a PAR2–arrestin1 complex, and arrestin2 colocalizes with the regulatory p85 PI3K subunit within the pseudopodia of cells during PAR2-mediated chemotaxis, suggesting that the arrestin2–PI3K association may spatially restrict PI3K activity and that this localized inhibition PI3K may be involved in PAR2-stimulated chemotaxis.

4.5.3 Phosphatidylinositol 4-phosphate 5-kinase

Another lipid kinase, phosphatidylinositol 4-phosphate 5-kinase (PIP5K) 1α, regulates clathrin and AP2 function during clathrin-dependent GPCR endocytosis.[71] PIP5K1α produces phosphatidylinositol 4,5-bisphosphate (PIP2) on the inner leaflet of the clathrin-coated pit, promoting polymerization of clathrin and AP-2 and assembly of the clathrin coat. Arrestin3 recruits PIP5K1α to activated β2-adrenergic receptors, increasing PIP2 formation and enhancing receptor endocytosis.

5. CONCLUSIONS

Arrestins regulate a robust network of protein and lipid kinases and phosphatases that allow GPCRs to modulate several downstream signaling cascades not available to heterotrimeric G protein-regulated effectors. In functioning as ligand-regulated scaffolds, they control the timing and location of signaling, directing multifunctional enzymes, like ERK1/2 and PP2A, toward some substrates and away from others. In a curious parallel to their role in terminating G protein signaling, arrestins appear to provide tonic inhibition of some kinase pathways, like JNK3 and NF-κB, while at the same time enabling GPCR-catalyzed activation of others. Arrestin kinase/phosphatase scaffolds regulate cytoskeletal rearrangement, vesicle endocytosis and exocytosis, cell migration and chemotaxis, and transmit signals to the cell nucleus to control cell proliferation, apoptosis, and survival. As subsequent chapters in this volume will attest, arrestin-dependent kinase/phosphatase signaling is integrated into GPCR-regulated physiologic processes at multiple levels, and improving our understanding of these multifunctional scaffolds may provide keys to novel treatments for diseases as seemingly diverse as cancer, inflammation, osteoporosis, and mental illness.

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