β-Arrestin Scaffolding of Phosphatidylinositol 4-Phosphate 5-Kinase Iα Promotes Agonist-stimulated Sequestration of the β2-Adrenergic Receptor

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Members of the seven-transmembrane receptor (7TMR) superfamily are sequestered from the plasma membrane following stimulation both to limit cellular responses as well as to initiate novel G protein-independent signaling pathways. The best studied mechanism for 7TMR internalization is via clathrin-coated pits, where clathrin and adaptor protein complex 2 nucleate and polymerize upon encountering the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to form the outer layer of the clathrin-coated vesicle. Activated receptors are recruited to clathrin-coated pits by β-arrestins, scaffolding proteins that interact with agonist-occupied 7TMRs as well as adaptor protein complex 2 and clathrin. We report here that following stimulation of the β2-adrenergic receptor (β2-AR), a prototypical 7TMR, β-arrestins bind phosphatidylinositol 4-phosphate 5-kinase (PIP5K) Iα, a PIP2-producing enzyme. Furthermore, β-arrestin2 is required to form a complex with PIP5K Iα and agonist-occupied β2-AR, and β-arrestins synergize with the kinase to produce PIP2 in response to isoproterenol stimulation. Interestingly, β-arrestins themselves bind PIP2, and a β-arrestin mutant deficient in PIP2 binding no longer internalizes 7TMRs, fails to interact with PIP5K Iα, and is not associated with PIP kinase activity assayed in vitro. However, a chimeric protein in which the core kinase domain of PIP5K Iα has been fused to the same β-arrestin mutant rescues internalization of β2-ARs. Collectively, these data support a model in which β-arrestins direct the localization of PIP5K Iα and PIP2 production to agonist-activated 7TMRs, thereby regulating receptor internalization.

The seven-transmembrane receptors (7TMRs) are integral membrane proteins defined by seven α-helical membrane-spanning segments and comprise the largest group of mammalian cell surface receptors with nearly 1000 members (1, 2). This superfamily is responsive to diverse stimuli, with individual receptors responding to hormones, neurotransmitters, and odorants among other signals. Although the signaling cascades initiated by these receptors are critical for intracellular responses to changes in the extracellular environment, termination of signal transduction and proper regulation of responses to stimuli are equally important for proper cellular function. Failures in the regulation of these pathways can lead to undesirable physiological consequences including tumorigenesis (3), vascular hypertrophy (4), and heart failure (4, 5). The agonist-specific process by which 7TMRs are silenced is a highly conserved, two-phase process known as homologous desensitization (6). The agonist-occupied 7TMR is phosphorylated on intracellular serine and threonine residues by a G protein-coupled receptor kinase (7), followed by the recruitment of arrestin proteins (rod and cone arrestins in the visual system and the ubiquitously expressed isoforms β-arrestin1 and β-arrestin2) to the G protein-coupled receptor kinase-phosphorylated receptors. Binding of arrestins sterically occludes the sites of receptor interaction with heterotrimeric G proteins and limits the responsiveness of the 7TMR to repeated stimulation (8, 9). Furthermore, recent studies have shown β-arrestins scaffold multiple isofoms of phosphodiesterases (10) and diacylglycerol kinases (DGKs) (11), enzymes that metabolize the second messenger molecules cAMP and diacylglycerol, respectively, further enhancing the efficiency of arrestin-mediated 7TMR signal quenching.

Although desensitization of 7TMRs begins silencing G protein signaling within seconds of activation, it has been well established that over the span of several minutes, agonist-occupied receptors are also internalized from the cell surface (12). This sequestration not only confines the receptor in a cellular compartment inaccessible to extracellular ligands and membrane-associated G proteins but also serves to initiate processes such as receptor dephosphorylation and recycling or proteolytic degradation (1, 13). In addition, 7TMR internalization is also required for various signaling pathways, including the
β-arrestin-dependent, G protein-independent activation of mitogen-activated protein kinases (14-17). 7TMRs are capable of internalization by various mechanisms. However, the best studied pathway for receptor endocytosis is via clathrin-coated pits, where β-arrestins serve as multivalent adaptor proteins to connect the 7TMR cargo to clathrin (18) and the β2 adaptin subunit of the heterotetrameric AP-2 adaptor complex (19, 20). β-Arrestins are integral components of this process as demonstrated by the severe impairment of 7TMR internalization in tissues from β-arrestin knock-out mice (21) as well as cells transfected with siRNA to deplete β-arrestin expression (22). Moreover, this endocytosis requires the interaction of β-arrestins with membrane phospholipids, particularly PIP₂ and PIP₃. Previous experiments showed that elimination of the β-arrestin2 phosphoinositol-binding pocket by the mutation of three basic residues created a mutant protein (β-arrestin2 RRK/Q) indistinguishable from wild type β-arrestin2 with respect to interactions with 7TMRs and clathrin but that did not internalize activated receptors (23).

Formation of a clathrin-coated pit begins with a nucleation event involving AP-2 and clathrin with PIP₂, concentrations of which are elevated in the plasma membrane (24, 25). As its outer clathrin layer begins to polymerize, the budding vesicle incorporates additional PIP₂-binding proteins involved in trafficking and endocytosis, including AP180/CalM (26), epsin (27), and the large GTPase dynamin (28). The importance of PIP₂ in these events makes its production by type I PIP5Ks a key mechanism for enhanced PIP2 production via co-localization of enzymes, we questioned whether may affect the PIP5K I

EXPERIMENTAL PROCEDURES

Materials and Reagents—Tissue culture reagents and Lipofectamine 2000 transfection reagent were purchased from Invitrogen. The radiolabeled compounds [³H]CGP-12177, [γ-³²P]ATP, and [³²P]orthophosphate were purchased from PerkinElmer Life Sciences. Chemically synthesized siRNAs were from Dharmacon, and GeneSilencer transfection reagent was bought from Gene Therapy Systems. P(4)P and purified lipid standards were sold by Avanti Polar Lipids. Thin layer chromatography plates were from Whatman International Ltd. Site-directed mutagenesis was done using a QuikChange II kit bought from Stratagene. All other materials were purchased from Sigma.

DNA Plasmids—FLAG-β-arrestin2 RRK/Q-5-kinase domain fusion protein (RRK5K) was engineered by PCR with rat FLAG-β-arrestin2 RRK/Q as the template and primers replacing the stop codon with a HindIII restriction cut site. The PCR product was subsequently cut with XbaI and HindIII and ligated into pcDNA 3.1 neo + vector. The core kinase domain of PIP5K Iα (residues 59-438) was created by PCR with primers (5’-3’): AAAAAAGCTTGGTGGCGATCCCGCGGTCCCTTCC and AAAAAAGCTTCTAAACAAACCTGTAAGACTG, cut with HindIII, and inserted into the plasmid described above. DNA sequencing at the Duke University DNA Analysis Facility confirmed the finished plasmid encoding FLAG-β-arrestin2 RRK/Q followed by two glycine residues and the PIP5K Iα kinase domain with a terminal stop codon. Plasmid encoding His₆-Myc-tagged PIP5K Iα was graciously provided by Dr. Matt Topham at the Huntsman Cancer Institute, University of Utah. All other plasmids used were created previously in the Lefkowitz laboratory.

Cell Culture and Immunoprecipitation—HEK293 cell culture conditions, transient transfection, and SDS-PAGE were as described previously (22). Briefly, the cells were treated with isoproterenol or propranolol as indicated. The cells were washed once with phosphate-buffered saline at 4 °C and were harvested by gentle scraping, pelleted, and resuspended in glycerol lysis buffer including protease inhibitors. The lysates were normalized for equal protein concentrations and immunoprecipitated with polyclonal rabbit β-arrestin antibody (A1CT) that has been previously described (37). Immunoprecipitation reactions were incubated at 4 °C for 3 h, washed three times with glycerol lysis buffer, and then resuspended in SDS running buffer. The samples were subjected to SDS-PAGE analysis and Western blotting with polyclonal goat PIP5K Iα antibody (N-20; Santa Cruz Biotechnology).

In Vitro Binding Experiments—HEK293 cells in 10-cm culture dishes transfected with His₆-Myc-PIP5K Iα or empty vector were lysed in 1.0 ml of glycerol lysis buffer (5.0 mM HEPES, 250 mM NaCl, 0.5% Nonidet P-40, 10% glycerol) containing 0.1% maltoside and protease inhibitor tablets (Roche Applied Science). The cells were incubated with lysis buffer for 1 h at 4 °C for maximum solubilization of PIP5K Iα. Cell debris was cleared by centrifugation (20,000 × g for 20 min) and then tumbled with 10 μl of anti-Myc beads (Covance) for 1 h at 4 °C. Myc beads containing the bound PIP5K were then washed three
**RESULTS**

To test whether β-arrestins can interact with PIP5K Iα, HEK293 cells with endogenous complements of β-arrestin2, PIP5K Iα, and β2-AR were stimulated in a time course with 10 μM isoproterenol. With all proteins expressed at physiological levels, immunoprecipitation of β-arrestin2 with the anti-β-arrestin antibody A1CT (37) co-immunoprecipitated PIP5K Iα with isoproterenol stimulation showing a maximum effect (3.12 ± 0.26-fold increase in association) at 5 min (Fig. 1, A and B). A 10-min preincubation of the cells with 100 μM propranolol, a β2-AR antagonist, effectively blunted the isoproterenol response, verifying that the augmented association of β-arrestin and PIP5K Iα is specifically due to 7TMR stimulation. In support of this finding, FLAG epitope-tagged β-arrestin1 and 2 were overexpressed in HEK293 cells endogenously expressing PIP5K Iα. β-Arrestin immunoprecipitation showed a preferential binding of PIP5K Iα to FLAG-β-arrestin2 over FLAG-β-arrestin1, with both isoforms of β-arrestin interacting with PIP5K Iα at levels significantly above vector controls (Fig. 1, C and D). Furthermore, stimulation of endogenous β2-ARs with

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**FIGURE 1. PIP5K Iα co-immunoprecipitates with β-arrestins in HEK293 cells.** A, Western blots of endogenous β-arrestin immunoprecipitated (IP) with A1CT antibody and co-immunoprecipitated endogenous PIP5K Iα from HEK293 cells stimulated in a time course with 10 μM isoproterenol. Where indicated, the cells were preincubated for 10 min with 100 μM propranolol. Immunoblots (IB) shown are representative of three independent experiments. Statistical significance for time course samples was determined using a one-way ANOVA with a Bonferroni post-hoc test to correct for multiple comparisons (*, p < 0.05; **, p < 0.001). B, Immunoblots shown are representative of four independent experiments. Statistical significance for time course samples was determined using a one-way ANOVA with a Bonferroni post-hoc test to correct for multiple comparisons (*, p < 0.05; **, p < 0.001). C, Whole blots of FLAG immunoprecipitates and lysates from HEK293 cells endogenously expressing PIP5K Iα transfected with FLAG-β-arrestin1 or FLAG-β-arrestin2 as shown. Where indicated, endogenous β2-ARs were stimulated with 10 μM isoproterenol (ISO) for 5 min. Immunoblots shown are representative of four independent experiments. D, Quantification of PIP5K Iα co-immunoprecipitation with FLAG-β-arrestins. All of the data were normalized as percentages of the maximum observed co-immunoprecipitation of PIP5K Iα for each experiment. Statistical significance was determined using a one-way ANOVA with a Bonferroni post-hoc test to correct for multiple comparisons (*, p < 0.05; **, p < 0.001 versus nonstimulated control).
10 μM isoproterenol increased the amount of PIP5K Iα co-immunoprecipitated with FLAG-β-arrestin2 by an average of 2.46 ± 0.11-fold relative to nonstimulated vector control samples. Comparable results were observed for HEK293 cells treated with 50 μM carbachol acting through endogenous M1 muscarinic receptors (supplemental Fig. S1). FLAG-β-arrestin2 was also observed co-immunoprecipitating with Myc epitope-tagged PIP5K Iα in the same HEK293 experimental system, and the protein-protein interaction was enhanced by multiple 7TMR-stimulating compounds: thrombin, carbachol, and isoproterenol (supplemental Fig. S2). Taken together, these findings show that endogenous and overexpressed β-arrestins can interact with PIP5K Iα in living cells, and their association can be potentiated through 7TMR stimulation.

Although the data indicate that β-arrestin and PIP5K Iα can be found together in protein complexes isolated from cells, they do not address whether this interaction is direct or indirect. Notably in this regard, PIP5K Iα is capable of interacting with DGKζ (34) and AP-2 (39), both of which are known β-arrestin binding partners. Therefore, we screened a panel of β-arrestin2 deletion mutants to map regions required to bind PIP5K Iα and compared them with those previously published for other β-arrestin interactors. Interestingly, endogenous PIP5K Iα was observed co-immunoprecipitating with elements of both the N- and C-terminal halves of β-arrestin2 in addition to the full-length protein (Fig. 2A). This stands in contrast to the DGKs and AP-2, binding partners common to both β-arrestin and PIP5K Iα, which map to discrete loci in the primary sequence (Fig. 2B). To directly test the hypothesis that β-arrestin2 and PIP5K Iα can interact directly, we affinity-purified Myc-tagged PIP5K Iα from HEK293 cells and incubated these PIP5K immunoprecipitates with purified recombinant

FIGURE 2. β-Arrestin2 directly binds PIP5K Iα and is independent of association with DGKζ, AP-2, or clathrin. A, Western blots of endogenous PIP5K Iα (Upper and Middle panels) co-immunoprecipitated (IP) in FLAG immunoprecipitates from HEK293 cells were transfected with empty vector (pcDNA3), full-length FLAG-β-arrestin2, or FLAG-tagged β-arrestin2 truncation mutants (lower panel). The images shown are representative of three independent experiments. B, table illustrating β-arrestin2 amino acid residues required for interacting with previously mapped binding partners. C, quantification of purified recombinant β-arrestin2 co-immunoprecipitating with His6-Myc-PIP5K Iα affinity-purified from HEK293 cells. The data from three independent experiments were normalized as percentages of the maximum amount of β-arrestin2 detected by A1CT antibody for each experiment. Statistical significance was determined using a one-way ANOVA with a Bonferroni post-hoc test to correct for multiple comparisons (***, p < 0.001, versus PIP5K Iα immunoprecipitates without IP6). Inset, representative A1CT immunoblot (IB) of purified β-arrestin2 co-immunoprecipitated under the experimental conditions.
**β-Arrestin Scaffolding of PIP5K Enhances β2-AR Endocytosis**

### FIGURE 3. Interaction of PIP5K Iα with the β2-AR is agonist- and β-arrestin2-dependent.

**A** Western blots of FLAG immunoprecipitates (IP) with dithiobis(succinimidyldipropionate cross-linking from HEK293 cells stably expressing FLAG-β2-AR transfected with either control (CTL) or β-arrestin2-specific siRNA (βarr2). Immunoblots (IB) were probed for endogenous PIP5K Iα co-immunoprecipitating with the receptor and subsequently reprobed with A1CT and β2-AR antibodies. The cells were stimulated for 5 min with 10 μM isoproterenol as indicated. The images shown are representative of three independent experiments. **B** Densitometry quantification of receptor-associated PIP5K Iα with data normalized to nonstimulated control samples. Statistical significance was determined using a one-way ANOVA with a Bonferroni post-hoc test to correct for multiple comparisons (**, p < 0.01 versus nonstimulated control). RNAi, RNA interference.

**β-arrestin2 in vitro** (Fig. 2C). A 5-fold increase in β-arrestin2 binding was observed when incubated with Myc-tagged PIP5K Iα as opposed to control immunoprecipitates from cells transfected with empty vector. Furthermore, the interaction was effectively blocked by the inclusion of inositol hexaphosphate (IP₆) in the binding buffer at an equimolar concentration with β-arrestin2. This latter finding is similar to previously reported effects of IP₆ on the interactions of β-arrestins with 7TMRs and clathrin (23). Because β-arrestins are known to recruit enzymes such as Src (41) and DGKζ (11) into multi-protein signalosomes with activated receptors, we reasoned β-arrestin2 may similarly mediate the interaction of PIP5K Iα with 7TMRs. Endogenous PIP5K Iα co-immunoprecipitated with stably overexpressed β2-ARs in HEK293 cells in an agonist-dependent manner as demonstrated by dithiobis(succinimidyldipropionate cross-linking experiments, and this interaction is augmented 2.71 ± 0.51-fold by stimulation with isoproterenol (Fig. 3). Depletion of β-arrestin2 by RNA interference had no significant affect on the amount of PIP5K Iα associated with the receptor under basal conditions. However, isoproterenol treatment of the cells transfected with β-arrestin2 RNA interference failed to show agonist-stimulated recruitment of PIP5K Iα to the β2-AR (1.12 ± 0.17-fold of control basal), indicating a requirement for β-arrestin2 in facilitating this enzyme-receptor interaction. This phenomenon was also observed in cells transfected with a second siRNA targeting β-arrestin2, reinforcing the specificity of the RNA interference results (supplemental Fig. S3).

With the finding that β-arrestin2 is required to recruit PIP5K Iα to β2-ARs in response to agonist, we hypothesized that β-arrestins may affect isoproterenol-stimulated PIP5K activity and PIP₂ production in cells. Whole cell [³²P]orthophosphate labeling of HEK293 cells followed by lipid extraction and TLC separation showed that isoproterenol stimulation of endogenous β2-ARs yielded no net change in radiolabeled PIP₂ levels when β-arrestin2 and PIP5K Iα are expressed at endogenous levels (Fig. 4). Overexpression of either β-arrestin2 or PIP5K Iα alone also showed no significant effect on the radiolabeled pool of PIP₂ in either basal or stimulated samples. However, increasing expression of both proteins revealed a significant isoproterenol-stimulated elevation of [³²P]PIP₂ (2.1 ± 0.3-fold versus basal control cells). Similar results were seen with carbachol stimulation of endogenous M₁ muscarinic receptors in the same experimental system (supplemental Fig. S4). Thus, β-arrestin2 and PIP5K Iα show a synergistic effect on agonist-induced PIP₂ production in cells, consistent with the β-arrestin-mediated scaffolding of PIP5K Iα to activated receptors.

To further investigate the role of β-arrestins in PIP5K Iα activation and targeting, we examined the properties of a rat β-arrestin2 mutant with the amino acids Arg²¹⁴, Arg²¹⁸, and Lys²⁵² mutated to glutamine (RRK/Q). This is an orthologue of a previously described bovine β-arrestin2 mutant that is deficient in phosphoinositide binding but that retains the ability to
interact with clathrin and a 7TMR (rhodopsin) in vitro (23). However, despite binding receptors and clathrin equally as well as wild type β-arrestin2, RRK/Q was significantly impaired in its ability to direct receptor internalization when transfected into COS cells. This deficiency was attributed to PIP2 and PIP3 providing additional sites of membrane attachment for β-arrestins recruited to 7TMRs. However, when we tested the RRK/Q mutant for interaction with PIP5K I, it did not show the agonist-stimulated interaction exhibited by the wild type protein (Fig. 5, A and B). Even more dramatically, when FLAG-β-arrestin2 and FLAG-RRK/Q immunoprecipitates were assayed for endogenous co-immunoprecipitated PI(4)P kinase activity in vitro, wild type β-arrestin2 was associated with robust kinase activity (20.0 ± 4.0-fold over vector control), whereas β-arrestin2 RRK/Q was indistinguishable from the vector control (2.1 ± 0.7-fold increase) (Fig. 5C).

These data suggested that rather than elimination of a secondary membrane attachment point, a defect in receptor localized of PIP2 production and subsequent loss of PIP2-mediated processes such as clathrin-coated pit formation may be responsible for the inability of the mutant to internalize 7TMRs. Seeking to test this hypothesis, we constructed a chimeric protein with FLAG-RRK/Q fused to the core catalytic domain of PIP5K I. This construct, named RRK5K, was expressed in HEK293 cells, and unlike the RRK/Q mutant, FLAG immunoprecipitates of RRK5K promoted significant32P phosphorylation of PI(4)P in vitro, indicating that the fusion protein possessed PIP5K catalytic activity as intended (supplementalFig. S5).

We next sought to determine whether the incorporation of PIP5K activity in the RRK5K mutant was sufficient to rescue the loss of 7TMR internalization reported for the β-arrestin2 RRK/Q. HEK293 cells stably expressing the β2-AR were transfected with siRNA targeting the endogenous human β-arrestin2 and co-transfected with rat β-arrestin2-GFP, RRK/Q-GFP, or RRK5K (upper panel). Internalization for each sample was quantified as the percentage of loss of [H]CGP-12177 binding after 30 min of stimulation with 10 μM isoproterenol. The data shown are the averages ± S.E. of three independent experiments, and statistical significance was determined using a one-way ANOVA with a Bonferroni post-hoc test to correct for multiple comparisons (*, p < 0.05 versus pcDNA3 control).

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structures used originated from rat β-arrestin2 and consequently were unaffected by siRNA directed against human β-arrestin2 (Fig. 5D, upper panel). These samples, either nonstimulated or isoproterenol-treated, were incubated with [3H]CGP-12177, a hydrophilic β2-AR ligand, and assayed under whole cell saturation binding conditions. Receptor internalization was defined in these experiments as an isoproterenol-stimulated loss of cell surface bound [3H]CGP relative to the nonstimulated counts in these experiments as an isoproterenol-stimulated loss of cell surface bound [3H]CGP relative to the nonstimulated counts for each transfection condition. Cells transfected with empty vector showed low levels of β2-AR internalization (7.1 ± 3.6%) consistent with previous β-arrestin2 RNA interference experiments (22), whereas expression of β-arrestin2-GFP enhanced sequestration to 27.8 ± 2.7%. As described previously (23), receptor endocytosis in RRK/Q-transfected cells was negligible with 3.3 ± 4.8% internalization. However, cells expressing the RRK5K mutant exhibited robust β2-AR sequestration (33.0 ± 6.7%), suggesting that the chimeric fusion of PIP5K activity with the RRK/Q mutant is sufficient to circumvent the defective interaction with PIP5K Iα and rescue β2-AR endocytosis. In a larger context, these findings also illustrate the importance of the interaction of β-arrestin with phosphoinositides and how this may affect protein-protein interactions and other β-arrestin-mediated processes.

**DISCUSSION**

Both β-arrestins and PIP2 produced by the PI(4)P 5-kinase family of enzymes are required for the efficient endocytosis of agonist-stimulated 7TMRs via clathrin-coated pits. In this report we have shown that β-arrestins (preferentially β-arrestin2) bind PIP5K Iα and mediate the association of PIP5K Iα with β2-ARs, with both processes potentiated by 7TMR stimulation. Also, as evidenced by the deficiencies of the RRK/Q mutant, formation of the β-arrestin-PIP5K Iα complex is dependent on the ability of β-arrestin to interact with PIP2/3, and disruption of this association effectively eliminates 7TMR internalization. Collectively, these data suggest a positive feedback mechanism where β-arrestin binding to phosphoinositides enhances 7TMR internalization by putting β-arrestin in a conformation to promote the interaction with PIP5K Iα consequently elevating local PIP2 concentrations (Fig. 6). It is also likely that other β-arrestin-associated signaling such as ARF6 and phosphatidic acid produced by DGKs further up-regulate PIP5K Iα enzymatic activity. Recent data by Kawasaki et al. (42) showed that an interaction between AP-2 and catalytically active DGKδ are required for efficient clathrin-mediated endocytosis in HeLa and COS7 cells. These findings also support a model for receptor internalization where clathrin-coated pit formation is triggered by DGK-mediated up-regulation of PIP5K activity in a process scaffolded by β-arrestins and the endocytic machinery. Thus, it appears the β-arrestins have adopted multiple roles in mediating internalization of stimulated receptors, connecting 7TMRs to the endocytic machinery and coordinating PIP5K Iα localization and activity.

As mentioned above, in the whole cell radiolabeled lipid extracts (Fig. 4), there was no change in the bulk PIP2 levels observed with isoproterenol stimulation when β-arrestins and PIP5K Iα were expressed endogenously. However, this result is not unexpected when the presence of endogenous phosphoinositide 5-phosphatases are taken into consideration. These enzymes, for which there currently are no pharmacological inhibitors, can counteract agonist-stimulated PIP5K activity to maintain homeostasis. The observation that overexpression of both β-arrestin2 and PIP5K Iα are required for agonist-stimulated PIP2 synthesis, whereas overexpression of either protein alone had no effect, would argue that efficient targeting and allosteric activation of PIP5K are equally as important as increasing kinase expression to overcome repression by the endogenous 5-phosphatases. Furthermore, we propose that
7TMR-stimulated PIP₂ changes do occur under physiological conditions but on a local scale at macromolecular receptor signalingomes rather than the global lipid pool.

The discovery of PIP5K ια as a novel β-arrestin-binding partner also generates interesting hypotheses for future study. In addition to regulating the interaction of clathrin and AP-2, PIP₂ can promote actin polymerization (43, 44) as well as stimulate WASP, ARP2/3, and coflin and activate many other key proteins regulating cytoskeletal rearrangement (reviewed in Ref. 45). PIP₂ also serves as a substrate for phosphatidylinositol 3-kinase to produce PIP₃, which promotes anti-apoptotic signaling and is required for chemotaxis (46). Interestingly, β-arrestin has been implicated as a signaling node in both of these processes. β-Arrestin-dependent chemotaxis has been demonstrated for many chemokine 7TMRs including CXCR4 (47), PAR-2 (48), and the AT₁₄R (49). In the latter case, the β-arrestin chemotactic pathway was shown to be independent of G protein activity. Finally, β-arrestin-dependent events are required for signaling pathways both upstream and downstream of phosphatidylinositol 3-kinase. β-Arrestin1 is absolutely required for IFG-1-stimulated PI3K activity in mouse embryonic fibroblast cells (50), whereas β-arrestin2 influences dopaminergic behaviors by promoting inactivation of Akt, the best studied PIP₃ effector, by scaffolding Akt with its negative regulator, protein phosphatase 2a (51). Future investigation will determine whether any or all of these signaling pathways require β-arrestin-scaffolded PIP5K activity and receptor-localized PIP₂ production.

As the lists of β-arrestin binding proteins and signaling pathways continue to expand at a very rapid pace, there is a growing appreciation for how β-arrestin conformational changes govern interactions with binding partners. In this regard, β-arrestin post-translational modification or allosteric binding of a cofactor can alter the conformation of β-arrestin into one or more “active” states, with increased affinity for a particular protein-protein interaction (38). Precedents for this method of regulation have been previously shown for β-arrestin phosphorylation and ubiquitination, which can modulate β-arrestin interactions with clathrin and the β₂-AR, respectively (52, 53). The finding described here that a β-arrestin interaction with phosphoinositides is required for PIP5K ια binding constitutes a novel β-arrestin regulatory mechanism. If in fact phosphoinositide binding alters β-arrestin conformation, additional binding partners may show different affinities for this state as opposed to the “inactive” β-arrestin. Moreover, in our in vitro binding experiments, IP₆₅ was a potent inhibitor of the β-arrestin2-PIP5K ια interaction. These findings are consistent with previous work by Gaidarov et al. (23) showing bidirectional regulation of β-arrestin binding to clathrin and 7TMRs by IP₆ (inhibitory) and PIP₃ (stimulatory), respectively. Based on the data presented here, we propose that PIP5K ια is another binding partner regulated by β-arrestin binding to either IP₆₅ or PIP₂₆ and that reciprocal regulation by inositol polyphosphates and phosphoinositides may be a general mechanism for modulation of additional β-arrestin interactions. Further experiments with the β-arrestin2 RRQ/Κ mutant in the future will likely help define the physiological consequences of IP₆₅, PIP₂₆, and PIP₃ binding on β-arrestin functions.

Acknowledgments—We thank Dr. Matt Topham for comments on the manuscript. We also thank Donna Addison and Elizabeth Hall for excellent secretarial assistance.

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