# Characterization of β-arrestin-modulated Lipid Kinase Activities for Diacylglycerol and Phosphatidylinositol 4-phosphate

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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# Abstract

The study of arrestins as regulators of seven transmembrane receptor (7TMR) signaling has revealed multiple levels of complexity, initiating desensitization of G protein activity and coordination of receptor internalization via clathrin-coated pits. Recently, β-arrestins have also been shown to act as adaptor proteins, mediating G protein-independent signaling as well as scaffolding of enzymes that degrade second messenger molecules. This latter function was demonstrated by β-arrestins recruiting PDE4 phosphodiesterase to $G_s$-coupled $\beta_2$-adrenergic receptors, enhancing metabolism of the second messenger cAMP. As β-arrestins universally interact with members of the 7TMR superfamily, we sought to determine if this phenomenon of concerted desensitization might be applicable to additional receptor subtypes.

We screened for β-arrestin-binding proteins among modulators of diacylglycerol and $IP_3$ (second messengers downstream of $G_q$-coupled 7TMRs). We observed β-arrestins constitutively interacted with members of the diacylglycerol kinase (DGK) family, which phosphorylate diacylglycerol to create phosphatidic acid. Furthermore, examining lipid extracts of $^{32}P$ labeled cells separated by TLC, we observed that overexpression of β-arrestin enhanced phosphatidic acid (PA) production after M1 muscarinic receptor stimulation. Conversely, depletion of β-arrestins by RNA

interference showed significantly decreased agonist-stimulated PA accumulation. Additionally, overexpression of a β-arrestin2 mutant that binds DGKs but not receptors served as a dominant negative for agonist-dependent DGK activity. These results demonstrate a requirement for β-arrestins in DGK translocation to the membrane, and specifically to activated 7TMRs, where concentrations of second messengers are at their highest.

Phosphatidic acid is an effector for several enzymes, including the phosphatidylinositol 5-kinases (PIP5K), which phosphorylate PIP to make $PIP_2$. Thus, we hypothesized β-arrestin-targeted DGKs may regulate PIP5K activity. PIP5K Iα associated with β-arrestin2 in an agonist-dependent manner in HEK293 cells, and a β-arrestin2 mutant defective in receptor endocytosis (a $PIP_2$-dependent function) was impaired. Furthermore, knockdown of β-arrestin2 by RNAi significantly decreased the amount of PIP5K Iα detected in receptor immunoprecipitates. In TLC assays, overexpressing both β-arrestin2 and PIP5K Iα enhanced agonist-stimulated $PIP_2$ labeling, while either protein alone had no effect. These data support the concept of β-arrestin binding to 7TMRs and enriching local membrane concentrations of PA, which then stimulates production of $PIP_2$, promoting receptor internalization.

# Contents

# List of Tables

# List of Figures

# List of Abbreviations

| | |
|---|---|
| **7TMR** | Seven transmembrane receptor |
| **AC** | Adenylyl Cyclase |
| **AMP** | Adenosine monophosphate |
| **ANOVA** | Analysis of variance |
| **ATII** | Angiotensin II |
| **$AT_{1A}R$** | Angiotensin II type 1A receptor |
| **ARF6** | ADP-ribosylation factor 6 |
| **ARNO** | ARF nucleotide binding site opener |
| **ATP** | Adenosine triphosphate |
| **$\beta_2$-AR** | $\beta_2$-adrenergic receptor |
| **βarr** | β-arrestin |
| **βarr0N** | β-arrestin 2 no N-terminus |
| **cAMP** | 3′-5′ cyclic adenosine monophosphate |
| **CBC** | Carbachol |
| **CRD** | Cysteine-rich domain |
| **DAG** | Diacylglycerol |
| **DGK** | Diacylglycerol kinase |
| **DSP** | Dithiobis(succinimidyl)propionate |

| | |
|---|---|
| **DTME** | Dithio-bis-maleimidoethane |
| **EGF** | Epidermal growth factor |
| **ERK** | Extracellular signal-related kinases |
| **FACS** | Fluorescence activated cell sorting |
| **GDP** | Guanosine diphosphate |
| **GEF** | Guanine nucleotide exchange factor |
| **GFP** | Green fluorescent protein |
| **GPCR** | G protein coupled receptor |
| **G protein** | Guanine nucleotide binding protein |
| **GRK** | G protein-coupled receptor kinase |
| **GTP** | Guanosine triphosphate |
| **H1R** | Histamine H-1 receptor |
| **IBMX** | Isobutyl methylxanthine |
| **IGF-1** | Insulin-like growth factor 1 |
| **$IP_3$** | Inositol 1,4,5-triphosphate |
| **Iso** | Isoproterenol |
| **JNK3** | Jun N-terminal kinase 3 |
| **LPA** | Lysophosphatidic acid |
| **M1R** | M1 Muscarinic Receptor |
| **MAP** | Mitogen activated protein |

| | |
|---|---|
| **MARCKS** | Myristoylated alanine-rich C-kinase substrate |
| **MEF** | Mouse embryonic fibroblast |
| **MKK** | MAP kinase kinase |
| **MKKK** | MAP kinase kinase kinase |
| **PA** | Phosphatidic acid |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PAR** | Protease-activated receptor |
| **PBS** | Phosphate-buffered saline |
| **PDBu** | Phorbol 12,13-dibutyrate |
| **PDE** | Phosphodiesterase |
| **PI** | Phosphatidylinositol |
| **PI3K** | Phosphatidylinositol 3-kinase |
| **PIP** | Phosphatidylinositol 4-phosphate |
| **$PIP_2$** | Phosphatidylinositol 4,5-bisphosphate |
| **$PIP_3$** | Phosphatidylinositol 3,4,5-trisphosphate |
| **PIP5K** | Phosphatidylinositol 4-phosphate 5-kinase |
| **PKA** | Protein Kinase A |
| **PKC** | Protein Kinase C |
| **PKD** | Protein Kinase D |
| **PLC** | Phospholipase C |

| | |
|---|---|
| **PMA** | Phorbol 12-myristate 13-acetate |
| **RGS** | Regulators of G protein signaling |
| **RNAi** | RNA interference |
| **RRK/Q** | β-arrestin 2 R233Q, R237Q, K251Q triple mutant |
| **RTK** | Receptor tyrosine kinase |
| **SDS** | Sodium dodecyl sulfate |
| **S.E.** | Standard error |
| **SH** | Src Homology |
| **siRNA** | Small interfering RNA |
| **SJ-1** | Synaptojanin-1 |

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# 1. Introduction

## *1.1 Conventional Seven Transmembrane Receptor Signaling*

The ability to sense and adapt to the surrounding environment is arguably the most fundamental requirement for maintaining homeostasis in all living beings. For a cell to thrive in changing surroundings it must be able to convert extracellular cues to intracellular responses and for eukaryotic cells, many of these signals are transduced across the plasma membrane by seven transmembrane receptors (7TMRs). The 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 one thousand members [1, 2]. This receptor superfamily is responsive to widely varying stimuli, including hormones, neurotransmitters, odorants and light. Despite this diversity, these receptors demonstrate fundamentally conserved signal transduction processes. In response to agonist stimulation the 7TMR alters its intracellular conformation to promote activation of one or more heterotrimeric guanine nucleotide binding proteins (G proteins). The association of a G protein with a 7TMR (also known as a G protein coupled receptor or GPCR) causes the Gα subunit to exchange its bound GDP molecule for GTP [3-5]. This new conformation leads to dissociation of the inactive G protein heterotrimer producing a free Gα subunit and Gβγ heterodimer. Both Gα and Gβγ are capable of interacting with specific effector proteins to stimulate the generation

of second messenger molecules involved in intracellular signaling cascades (Figure 1-1) [6-11]. This chain of transduction also promotes signal amplification, as each step is catalytic: one agonist-occupied receptor can activate multiple G proteins leading to exponential amplification at the levels of G protein effectors and second messengers.

While their composition *in vivo* is combinatorially complex, with 21 Gα, 6 Gβ, and 12 Gγ subunits reported to be expressed in human cells, G proteins are typically classified into four groups based on α subunit sequence homology: $G_s$, $G_i$, $G_q$, and $G_{12/13}$ (Table 1) [4]. Classically, $G_s$ stimulates the family of adenylyl cyclase (AC) enzymes, producing 3′-5′ cyclic adenosine monophosphate (cAMP), whereas $G_i$ is inhibitory for adenylyl cyclase activity, thus, lowering intracellular cAMP concentrations [12]. Because cAMP is an allosteric modulator of protein kinase A (PKA), among other enzymes, the activation of $G_s$ and $G_i$ by 7TMRs impacts a host of cellular processes dependent on tonic and agonist-stimulated PKA phosphorylation. For 7TMRs that couple to $G_q$, signal transduction proceeds through the stimulation of phospholipase C (PLC) and hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to produce the second messengers inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG) [13, 14]. The increase DAG concentration in the plasma membrane and the $IP_3$-dependent release of $Ca^{2+}$ from the endoplasmic reticulum are required events for the activation of protein kinase C (PKC) and protein kinase D (PKD) [15, 16]. Finally, the $G_{12/13}$ family of G proteins is most often

associated with activation of the small GTPase protein Rho through the guanine nucleotide exchange factor p115RhoGEF, thereby influencing actin cytoskeletal dynamics and cell adhesion [17].

**Table 1-1: Classification and Signaling Pathways of G proteins**

| | $G_s$ | $G_i$ | $G_{q/11}$ | $G_{12/13}$ |
|---|---|---|---|---|
| Gα Subunits | $G\alpha_s$, $G\alpha_{olf}$ | $G\alpha_i$, $G\alpha_o$, $G\alpha_t$, $G\alpha_{gust}$, $G\alpha_z$ | $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ | $G\alpha_{12}$, $G\alpha_{13}$ |
| Primary Effectors | AC ↑ | AC ↓, PLC ↑ (via Gβγ) | PLC ↑ | p115RhoGEF, PDZ-RhoGEF |
| Small Molecule Second Messengers | cAMP | | $IP_3$, DAG | |
| Secondary Effectors | PKA | | PKC, PKD | Rho |

**Figure 1-1: Conventional Seven Transmembrane Receptor Signaling.** The binding of a specific ligand to the extracellular face of its cognate receptor (Upper panel) causes an alteration in the intracellular 7TMR conformation, represented by a change from R to R*. This "active" conformation then couples with one or more heterotrimeric G proteins (Middle panel) and promotes the exchange of GTP for GDP on the α subunit. This nucleotide exchange dissociates Gα from Gβγ (Lower panel), both of which are then free to activate effector proteins such as adenylyl cyclase or phospholipase C, triggering signaling cascades.

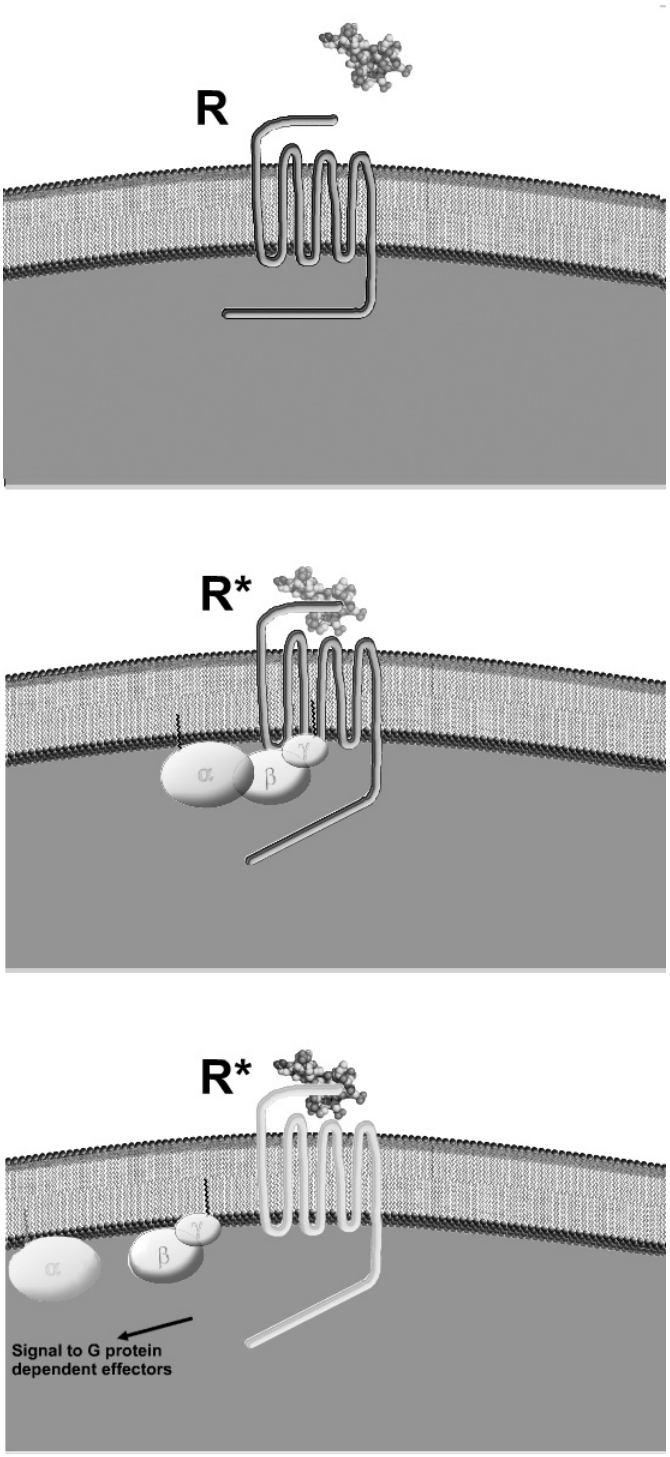


Figure 1-1: Conventional Seven Transmembrane Receptor Signaling

Ultimately the physiological consequences of G protein coupled receptor signaling is the sum of these signaling events. Adrenaline stimulation of adrenergic receptors in the heart promotes increases in heart rate and stroke volume. Release of inflammatory cytokines from sites of irritation or infection causes migration of white blood cells into the affected area. Neurotransmitters such as serotonin bind to their cognate receptors in neurons to trigger an electrochemical action potential. These are only a few examples of how activation of 7TMRs and G proteins lead to physiological responses [18-20]. Signaling pathways for processes such as cell growth, cell death, metabolism, membrane ion permeability, nucleic acid transcription, protein translation, and many more can also trace their initiation to stimulation of a 7TMR. Yet, as important as activation of these pathways is for initiating physiological responses, these signals must also be able to be turned off to maintain homeostasis.

## *1.2 Regulation of 7TMR Signaling Pathways*

Termination of signal transduction cascades and proper regulation of cellular responses to stimuli are fundamentally essential for cellular function. Conceptually, a 7TMR can be thought of as a molecular switch, turning on signaling in response to agonist. Before the system can be made to respond to a subsequent stimulus, the signals must be turned off and the switch reset. Failure to keep these signaling pathways in check also leads to undesirable physiological consequences including uncontrolled cell

growth and tumorigenesis [21], vascular hypertrophy [22], and heart failure [22, 23]. Inactivation of G proteins, degradation of second messengers and terminating receptor activation are all critical for proper cellular function.

### 1.2.1 Regulation of G protein Activity

Early studies of G proteins revealed an absolute requirement for GTP in the agonist-dependent increase in intracellular second messenger concentrations as well as the fact that the phosphorylation state of the Gα-bound guanine nucleotide governed this activity (Reviewed in [4, 5]). The GTP-bound Gα actively enhanced second messenger generation and was dissociated from Gβγ, while the GDP-bound protein was observed as an inactive heterotrimer. As discussed above, the agonist-occupied 7TMR conformation promotes the incorporation of GTP and G protein activation. The mechanism controlling the conversion of GTP to GDP, and thus the determinant of G protein deactivation, is the intrinsic GTPase activity of the α subunit. Following activation, the Gα begins the process of its own deactivation by cleaving the terminal phosphate bond of GTP. The GDP-bound Gα then re-associates with free Gβγ subunits, reforming an inactive complex.

However, while this process is sufficient for G protein inactivation, the Regulators of G protein Signaling (RGS) proteins greatly enhance the GTPase activity

and further accelerate G protein silencing. Studies with recombinant proteins *in vitro* have shown many RGS family members increase Gα catalytic activity by 40 fold over basal or more [24, 25]. Furthermore, in response to 7TMR stimulation, multiple members of the RGS family are known to increase their expression levels. RGS1 [26], RGS2 [27], and RGS16 [28] have been shown to induce protein translation after treatment of cells with the 7TMR agonists platelet activating factor, angiotensin II (ATII), and carbachol (CBC) respectively. This phenomenon constitutes a negative feedback loop; a common theme in receptor biology in which stimulation of a signaling pathway ultimately leads to signal termination via downstream effectors.

### 1.2.2 Metabolism of Second Messenger Molecules

Unlike the autocatalytic G proteins, most second messenger molecules require specific enzymes for inactivation. Traditionally the prototypical second messenger molecule of research, cAMP produced via $G_s$ activation of adenylyl cyclase is degraded by the family of cyclic nucleotide phosphodiesterases (PDEs) [29, 30]. The PDEs enzymatically break the cyclic ring of cAMP to produce adenosine monophosphate (AMP), which does not activate PKA or other cAMP effectors and has minimal biological activity. Furthermore, some PDE isoforms are targeted to specific subcellular localizations by virtue of interactions with signaling and adaptor proteins [31, 32]. The heterologous distribution of metabolizing enzymes allows for microdomains of this

second messenger, with the highest concentrations of cAMP localized at the membrane proximal to active AC [32, 33]. Thus, the amplitude and duration of cAMP signaling within a given cell, or even in a given microdomain, depends on the interplay between ACs and PDEs.

For 7TMRs that couple to $G_q$, second messenger degradation is an even more intricate process than the pathway for $G_s$-coupled receptors because the action of PLC produces two bioactive molecules ($IP_3$ and DAG) instead of one (cAMP). Once the $IP_3$ headgroup is cleaved from its lipid counterpart, it diffuses through the cytoplasm until it binds $IP_3$ receptors integrated into the endoplasmic reticulum [34]. This interaction triggers a burst of $Ca^{2+}$ efflux, altering the activities of calcium sensitive effectors such as calmodulin and calcineurin [35, 36]. The main route of $IP_3$ clearance is via sequential dephosphorylation to myo-inositol followed by re-incorporation into inositol lipids through the phosphoinositide cycle [37]. Alternatively, $IP_3$ can be further phosphorylated by inositol polyphosphate kinases to yield higher order inositol phosphates such as $IP_4$, $IP_5$, and $IP_6$ [38]. These inositol phosphate species no longer initiate calcium release, but rather, serve as signaling molecules in other cellular processes.

The main pathway of DAG metabolism is phosphorylation by the family of diacylglycerol kinases [39, 40]. To date, ten isoforms of mammalian DGK have been identified, including the recently discovered DGKκ [41]. All isoforms have at least two cysteine-rich C1 domains, homologous to the C1A and C1B motifs of protein kinase C, and a conserved catalytic domain. Further classification of DGKs into five subtypes is based on the presence of additional functional domains [42]. These enzymes are predominantly cytoplasmic, and translocate to the plasma membrane upon stimulation of many receptor types, including 7TMRs [43-45].

The reaction catalyzed by all DGKs is the ATP-dependent creation of phosphatidic acid through phosphorylation of the sn-3 position of DAG. The growing recognition of PA as an effector molecule in its own signaling pathways has elevated the roles of DGKs beyond simply antagonizing DAG signaling. PA influences vesicle trafficking, promotes translocation of Raf to the plasma membrane, and affects the activity of multiple enzymes including type I phosphatidylinositol 5-kinases [46, 47], PKCζ [48], and small GTPase proteins [49]. In many cases, the DGKs also physically associate with these targets, implicating the DGKs as the relevant PA source [47, 49].

### 1.2.3 Homologous and Heterologous Desensitization of 7TMRs

For the 7TMRs themselves, two pathways exist to quench or desensitize receptor activity. These pathways known as heterologous and homologous desensitization are classified based on their specificity for agonist-occupied receptors [1, 50]. Heterologous desensitization is the hyporesponsiveness of multiple 7TMR species in response to a single agonist. This process relies on a negative feedback loop where receptors are phosphorylated by the second messenger kinases PKA and PKC. As discussed above, 7TMR stimulation can increase the intracellular concentrations of cAMP ($G_s$-coupled receptors) or calcium and diacylglycerol ($G_q$-coupled receptors), which activate PKA and PKC, respectively. These kinases subsequently phosphorylate serine and threonine residues on a large number of 7TMRs (typically in the C-terminal tail, or second and third intracellular loops), negatively affecting receptor-G protein coupling (Figure 1-2, upper panels). In vitro reconstitution experiments have demonstrated either PKA or PKC phosphorylation of the $\beta_2$-AR decreases its ability to couple to $G_s$ by over 40% [51, 52]. This method of feedback inhibition affects both agonist-occupied, as well as unoccupied receptors containing consensus PKA- or PKC-dependent phosphorylation motifs, leading to a generalized decrease in responsiveness after an initial agonist treatment.

In contrast to non-specific heterologous desensitization, homologous desensitization silences only agonist-occupied 7TMRs in a highly conserved, two-phase manner. Typically, the agonist-occupied 7TMR is phosphorylated on serine and threonine residues in the C-terminal tail and intracellular loops by the G protein-coupled receptor kinases (GRKs) [50, 53]. However, GRK phosphorylation alone only attenuates 7TMR signaling between 0-30% depending on specific receptor and cell type [54-56]. The second phase of homologous desensitization is the stoichiometric binding of arrestin proteins to GRK phosphorylated 7TMRs. This phenomenon is not merely a result of general receptor phosphorylation: arrestins have shown a 100-fold preference for GRK-phosphorylated $\beta_2$-ARs over those phosphorylated by PKA as measured by G protein coupling assays [57]. Binding of arrestins sterically occludes the sites of receptor-G protein interaction; decreasing G protein activation by up to 80% and limiting the 7TMR's responsiveness to repeated stimulation (Figure 1-2, lower panels) [52, 54]. This mechanism is utilized by virtually the entire 7TMR superfamily, regardless of the G proteins activated by the receptors.

The versatility of the GRKs and arrestins is all the more remarkable when considering the extremely small number of proteins carrying out the process of desensitization. Of the seven-member GRK family in humans, GRK1 (also known as rhodopsin kinase) and GRK7 are exclusive to phototransduction pathways, while GRK4

**Figure 1-2: Mechanisms of Heterologous and Homologous Desensitization.**

Heterologous desensitization dampens multiple 7TMR species in response to agonist stimulation through second-messenger kinases such as PKA and PKC. In this example, stimulation of a $\beta_2$ adrenergic receptor ($\beta_2$AR) with isoproterenol (Iso) dissociates the heterotrimeric $G_s$ protein (Upper left panel). $G\alpha_s$ activates adenylyl cyclases, raising intracellular cAMP concentrations, which increases the activity of PKA. As many 7TMRs contain consensus PKA phosphorylation sites, both the ligand occupied $\beta_2$AR and non-stimulated receptor, in this case the histamine H-1 receptor (H1R) [58], are inhibited by this negative feedback mechanism (Upper right panel). In contrast, homologous desensitization is selective for 7TMRs in a ligand-induced active conformation. Using the same model system, Iso stimulation leads to $\beta_2$AR phosphorylation by one or more GRK proteins (Lower left panel), but not phosphorylation of the inactive H1R. β-arrestin then translocates to the plasma membrane where it preferentially interacts with the agonist-stimulated phospho-7TMR species, thereby occluding sites of G protein activation (Lower right panel).

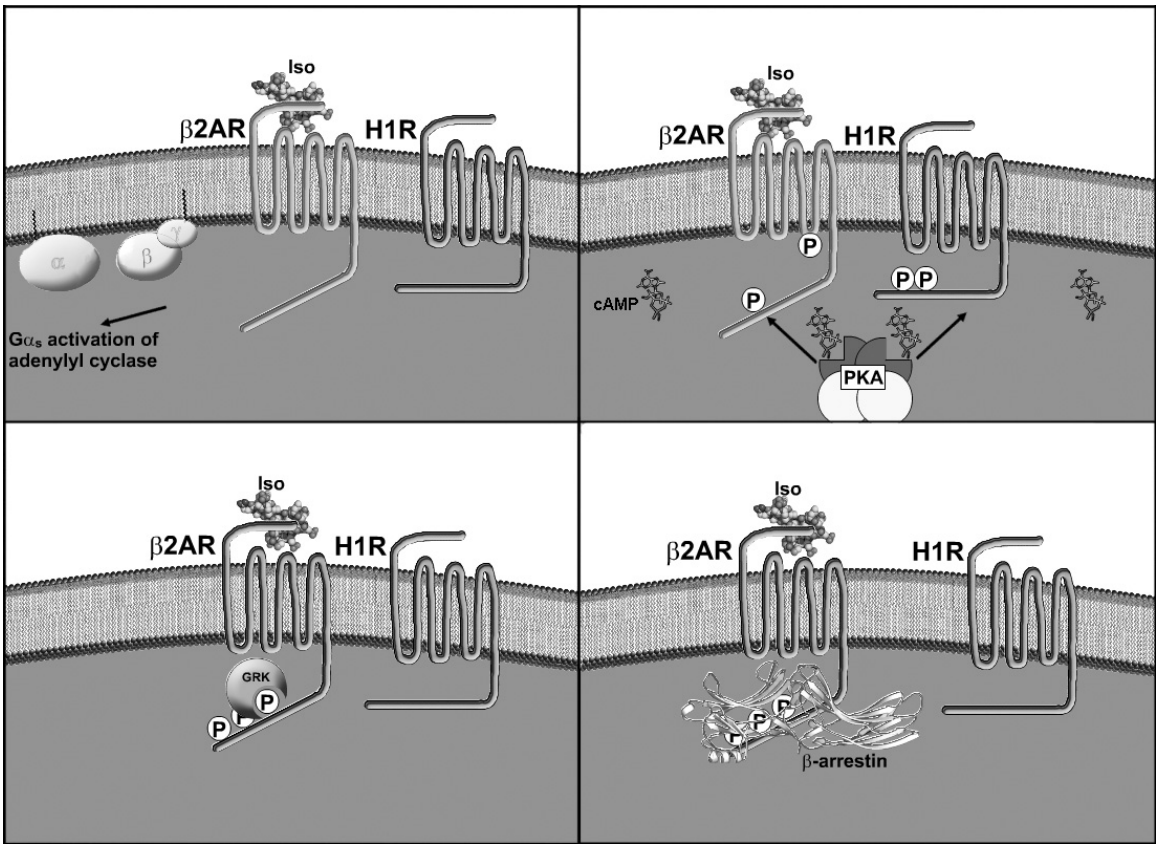


Figure 1-2: Mechanisms of Heterologous and Homologous Desensitization

shows a somewhat limited expression profile, leaving the widely expressed GRKs (GRK2, 3, 5 and 6) responsible for the majority of agonist-stimulated 7TMR phosphorylation [53, 59]. As with the GRKs, two of the four arrestin isoforms, visual arrestin and x-arrestin, are largely restricted to the rods and cones of the retina respectively [60, 61]. The ubiquitously expressed β-arrestin 1 and β-arrestin 2 mediate homologous desensitization in all other human cell types. Furthermore, it is now appreciated that β-arrestins also serve as multi-purpose scaffolding proteins for many intracellular signaling pathways in addition to antagonizing G protein signaling.

## *1.3 β-arrestins: Beyond 7TMR Desensitization*

In addition to their traditional role in homologous desensitization, recent studies have shown β-arrestins serve as adaptor proteins, enhancing signaling efficiency by co-localizing enzymes with their activators in multi-protein complexes or targeting proteins to specific subcellular locations [2, 62]. The list of non-receptor binding partners for β-arrestin continues to grow, with receptor sequestration, signaling cascades, ubiquitination, and second messenger metabolism pathways all scaffolded by β-arrestin (Table 1-2).

**Table 1-2: Partial List of Novel β-arrestin Binding Partners**. This figure contains many of the published non-G protein coupled receptor proteins interacting with β-arrestin 1 and 2. Also shown are preferential interactions of these binding partners with specific β-arrestin isoforms (if applicable), as well as observed functional consequences of β-arrestin scaffolding. Adapted from Lefkowitz, R.J. and S.K. Shenoy, *Transduction of receptor signals by beta-arrestins.* Science, 2005. **308**(5721): p. 512-7.

Table 1-2: Partial list of Novel β-arrestin Binding Partners

| Binding Protein | ß-Arrestin Isoform | Functional Consequences | Reference # |
|---|---|---|---|
| ***Trafficking Proteins*** | | | |
| Clathrin | ß-Arrestin 1, 2 | Endocytosis | 70, 73 |
| AP2 | ß-Arrestin 1, 2 | Endocytosis | 69, 74 |
| NSF | ß-Arrestin 1 | Endocytosis; recycling | 75 |
| ***Small G/GEFs*** | | | |
| ARF6 | ß-Arrestin 2>>1 | Endocytosis | 76 |
| ARNO | ß-Arrestin 2 | Endocytosis | 76 |
| Ral-GDS | ß-Arrestin 1, 2 | Cytoskeletal rearrangement | 63 |
| RhoA | ß-Arrestin 1 | Stress fiber formation | 64 |
| ***MAPK Cascade Components*** | | | |
| ASK1 | ß-Arrestin 1, 2 | JNK3 and p38 activation | 79 |
| c-Raf-1 | ß-Arrestin 1, 2 | ERK activation | 80 |
| JNK3 | ß-Arrestin 2>>1 | Stabilization of pJNK on endosomes | 79 |
| ERK2 | ß-Arrestin 1, 2 | Prolonged cytosolic pERK signaling | 2, 80 |
| ***Second Messenger Metabolizing Enzymes*** | | | |
| PDE4D family | ß-Arrestin 1, 2 | cAMP degradation | 108 |
| DGK family | ß-Arrestin 1, 2 | DAG metabolism; PA production | 65 |
| ***Non-Receptor Tyrosine Kinases*** | | | |
| c-Src | ß-Arrestin 1, 2 | Endocytosis, ERK activation | 72, 75 |
| Yes | ß-Arrestin 1 | Gaq activation and GLUT4 transport | 88 |
| Hck | ß-Arrestin 1 | Exocytosis of granules in neutrophils | 87 |
| Fgr | ß-Arrestin 1 | Exocytosis of granules in neutrophils | 87 |
| ***Others*** | | | |
| Mdm2 | ß-Arrestin 1, 2 | Ubiquitination, endocytosis | 77, 78 |
| IκBα | ß-Arrestin 1, 2 | Stabilization of IκBα upon ß2AR and TNFR stimulation | 104 |
| Dishevelled | ß-Arrestin 2 | Endocytosis of Frizzled4 | 94 |
| Smoothened | ß-Arrestin 2 | Gli-mediated transcription | 95 |
| PP2A | ß-Arrestin 1 | Dopaminergic behaviors | 105 |
| p300 | ß-Arrestin 1 | DNA transcription | 106 |
| CREB | ß-Arrestin 1 | DNA transcription | 106 |

### 1.3.1 β-arrestins and Clathrin-coated Pit Internalization

While 7TMR desensitization interdicts G protein signals within seconds of activation, it has become well established that over the span of several minutes, agonist-occupied receptors are also internalized from the cell surface [66]. The primary route for 7TMR endocytosis is via clathrin-coated pits [67]. Sequestration not only removes 7TMRs from the proximity of the membrane-associated G proteins, but also serves to initiate processes such as receptor dephosphorylation and recycling, and/or proteolytic degradation [68].

Work with purified proteins [69, 70] and mouse embryonic fibroblast (MEF) cells from β-arrestin knockout mice [71] have shown that β-arrestins play an important role in these processes. The degree of β-arrestin involvement in endocytosis is dependent on the amount and ratios of β-arrestins expressed in the experimental system, as well as such factors as the 7TMR species stimulated, cell type and passage number, and the cell's complement of GRKs [72]. β-arrestins, but interestingly not visual arrestins, bind the β2 adaptin subunit of the AP-2 adaptor complex [69] and all arrestins show a high affinity for clathrin. These proteins bind β-arrestins *in vitro* through LIEF (clathrin) [73] and RxR (AP-2) [74] amino acid motifs located within the C-terminal portion of β-arrestin 1 and β-arrestin 2. Mutation of these residues ablates 7TMR sequestration, but has no deleterious effects on β-arrestin translocation to the membrane or its ability to bind

GRK-phosphorylated 7TMRs. Additionally, factors enhancing receptor endocytosis such as N-ethylmaleimide-sensitive fusion protein (NSF) [75], the small GTPase ADP-ribosylation factor 6 (ARF6) [76] and its GEF the ARF nucleotide binding site opener (ARNO) [76] have been shown to utilize β-arrestins for their activation and plasma membrane targeting.

### 1.3.2 Ubiquitination of β-arrestins and 7TMRs

Following receptor sequestration, the 7TMR-containing endosome may either be recycled back to the plasma membrane or targeted for proteolytic degradation. Very often, proteins to be degraded are post-translationally modified on lysine residues by the attachment of the 76 amino acid protein ubiquitin, and this is also true for the $\beta_2$-AR. Iso-dependent ubiquitination and degradation of the receptor has been observed both *in vitro* and in cells and has also been shown to involve β-arrestin [77]. While wild type MEF cells show ubiquitinated β2-ARs after agonist stimulation, this modification is absent from β-arrestin 1/2 double knock-out MEFs. Restoring expression of β-arrestin 2, but not β-arrestin 1, in these cells restored wild type functionality. This ubiquitination event is carried out by a currently unknown ubiquitin ligase and is required for receptor degradation, but not internalization [77].

Furthermore, β-arrestins have been shown to directly interact with the E3 ubiquitin ligase MDM2 and are themselves ubiquitinated by this enzyme [77]. Interestingly, MDM2 ubiquitination does not appear to promote β-arrestin proteolysis. Rather, it is a required process for receptor internalization and appears to govern the strength of the β-arrestin-receptor interaction [78], consistent with an emerging role of ubiquitination modulating the signaling properties of some proteins in addition to promoting proteolytic degradation.

### 1.3.3 G protein-Independent Activation of MAP Kinases by β-arrestins

The role of β-arrestins as adaptor proteins in 7TMR endocytosis led to the discovery that they are also scaffolds for multiple signaling pathways. In this regard, the most extensively studied β-arrestin binding partners are the mitogen activated protein (MAP) kinases including Jun N-terminal kinase 3 (JNK3) [79] and the extracellular signal-related kinases (ERK) 1 and 2 [2, 80]. The MAP kinases are activated through phosphorylation, with MAP kinase kinase (MKK) enzymes as well as MAP kinase kinase kinase (MKKK) proteins creating a transduction cascade, enzymatically amplifying the signal at each level in the hierarchy. β-arrestins are necessary scaffolds that bring together several MAP kinase cascades such as Raf/MEK/ERK [80] and ASK/MKK4/JNK3 [79] through direct interactions with the MKKK and MAP kinase.

Furthermore, it has been shown, in particular for ERK, that this activation can occur via G protein-independent mechanisms as indicated through the use of 7TMR mutants uncoupled from G proteins, as well as synthetic peptide ligands which are biased agonists for β-arrestin recruitment over G protein signaling [81, 82]. ERK activated via β-arrestin also shows different temporal and spatial patterns of activation than those turned on by the G protein pathway. G protein activated ERK is distributed throughout the cell including the nucleus, where it phosphorylates transcription factors and is rapidly inactivated by nuclear ERK phosphatases [83]. In contrast, phospho-ERK activated by β-arrestin is more protracted and almost exclusively cytosolic, phosphorylating as yet unknown cytoplasmic substrates [83-85].

### 1.3.4 β-arrestin-scaffolded Src Family Non-Receptor Tyrosine Kinases

The pathways of internalization and ERK activation for some 7TMRs depend on an interaction between β-arrestin and members of the Src family of non-receptor tyrosine kinases. For Src [86], Hck [87], Fgr [87], and Yes [88] a Src homology 3 (SH3) domain binds a proline-rich sequence of β-arrestin and the SH1 catalytic domain of c-Src has also been shown to interact with an N-terminal region of β-arrestin 1 [89]. Thus, when β-arrestin is recruited to 7TMRs such as the $\beta_2$-AR or neurokinin-1 receptor, the Src family kinase is brought into a complex with the activated receptor [86]. There, Src can phosphorylate dynamin, the enzyme responsible for vesicle fission of clathrin-

coated pits from the plasma membrane, as well as clathrin, AP-2 and other endocytic elements [90]. Disrupting the β-arrestin-Src interaction or mutating the target dynamin tyrosine residues effectively block $\beta_2$-AR endocytosis.

β-arrestin-dependent Src activity has also been implicated in the process of transactivation, whereby 7TMR stimulation leads to signaling via a receptor tyrosine kinase (RTK) [91, 92]. In perhaps the clearest example of this phenomenon, β-arrestin-mediated recruitment of Src to the $\alpha_{2a}$-adrenergic receptor initiates metalloprotease-dependent "shedding" of heparin-binding epidermal growth factor (EGF)-like ligand, stimulating the EGF receptor in a paracrine fashion [93]. This transactivation pathway is likely responsible for some of the Ras-dependent ERK activation observed for many 7TMRs.

### 1.3.5 Additional Receptors Utilize β-arrestins

Perhaps even more surprising than the number of β-arrestin-scaffolded signaling pathways has been the discovery that β-arrestins not only bind classically G protein-coupled 7TMRs, but many other types of cell surface receptors as well. While the atypical 7TMRs Frizzled 4 and Smoothened do not demonstrably couple to G proteins for their developmental and chemotactic signaling, both receptors use β-arrestins for endocytosis. Wnt-stimulated Frizzled 4 internalizes when phosphorylated Dishevelled 2

bindins to β-arrestin 2 and escorts both proteins to the receptor [94]. Recruitment of β-arrestin 2 to Smoothened utilizes the more traditional pathway of receptor phosphorylation by GRK2 [95].

In addition to heptihelical receptors, the single transmembrane-spanning type III transforming growth factor-β receptor [96], low-density lipoprotein receptor [97], the nicotinic acetylcholinergic receptor ion channel [98], and the Na+/H+ antiporter NHE5 [99] have all been shown to internalize in a β-arrestin dependent manner. The insulin-like growth factor type I (IGF-1) receptor, a classical RTK, not only co-opts β-arrestin for receptor sequestration but β-arrestin signaling pathways as well. IGF-1-mediated anti-apoptotic effects via phosphatidylinositol 3-kinase (PI3K) [100] and phospho-ERK [101], as well as MDM2-dependent receptor ubiquitination [102, 103] have been shown to depend on β-arrestin 1 expression. Thus, while the 7TMRs constitute the single largest mammalian receptor family, the roles of the β-arrestins have grown to exceed even this scope to include signaling and endocytosis of additional membrane-spanning receptors and RTKs.

## *1.4 Concerted Desensitization is Directed by β-arrestins*

Until very recently, desensitization of 7TMRs signals could be viewed as a "passive" function of β-arrestins, binding GRK-phosphorylated receptors and

conveniently blocking G protein activation in the process. However, even traditional GPCR desensitization has required revision to include dynamic scaffolding roles for β-arrestin in light of the work by Perry, et al. that β-arrestin 1 and 2 bind the PDE4D family of phosphodiesterases [108]. In wild type, but not β-arrestin double knockout MEFs, membrane-associated PDE4D protein and phosphodiesterase activity increased with $\beta_2$-AR stimulation by isoproterenol in a time course consistent with β-arrestin-mediated recruitment. Replacement of β-arrestin 1 in the knockout MEFs rescued the observed PDE function, further validating this hypothesis. Further studies of the β-arrestin-PDE4D complex have also been shown to antagonize the PKA-mediated process of $\beta_2$-AR "switching" from $G_s$ coupling to $G_i$ coupling [109, 110]. As discussed in section 1.2.2, cAMP signaling is believed to be restricted to microdomains at the plasma membrane, activating localized pools of PKA near stimulated receptors. Recruitment of the β-arrestin-PDE complex to these active 7TMR sites therefore coordinates a process of "concerted desensitization" for $G_s$ and cAMP. The production of cAMP by is blocked by β-arrestin prohibiting further G protein stimulation of AC, but also the rate of second messenger clearance is accelerated by PDE localization in the proximity of cAMP microdomains.

## *1.5 Objectives for this Dissertation*

At the time of this dissertation, it has been established that for the $G_s$-coupled $\beta_2$-AR that β-arrestins are a concerted desensitization platform for both $G_s$ and cAMP via interaction with PDE4Ds. This targeted recruitment brings PDE into a protein complex with agonist-stimulated $\beta_2$-ARs, which are the cell's highest sites of AC activity and cAMP production. Thus, β-arrestins accelerate breakdown of cAMP and facilitate inactivation of the second messenger kinase PKA in addition to their traditional role of antagonizing G protein-7TMR coupling. However, this complex obviously is not relevant for the large number of $G_q$-coupled 7TMRs, as they do not use cAMP as a second messenger. Hypothesizing an analogous mechanism of concerted desensitization may exist for this receptor class (Figure 1-3), a large screen of $IP_3$ and DAG regulatory enzymes was undertaken, looking for novel β-arrestin binding partners. This screen has suggested an interaction between β-arrestins and multiple isoforms of DGK.

**Figure 1-3. Concerted Desensitization as a General Property of β-arrestins.** The work of Perry et al [108] established an interaction between β-arrestins and isoforms of PDE4D phosphodiesterase, showing this complex is recruited to Iso-stimulated $\beta_2$-ARs (upper panel). This localizes the PDE to microdomains of cAMP at the plasma membrane accelerating cAMP degradation. Coupled with the traditional role of β-arrestins in blocking G protein activation, this was the first reported example of a concerted mechanism of desensitization for $G_s$ proteins and second messengers. The central hypothesis of this dissertation is that analogous systems of concerted desensitization exist for other classes of 7TMRs such as $G_q$-coupled receptors (lower panel). If this is true, we expect β-arrestin to be responsible for agonist-dependent recruitment of regulatory enzymes for $IP_3$ and/or DAG to $G_q$-coupled 7TMRs.

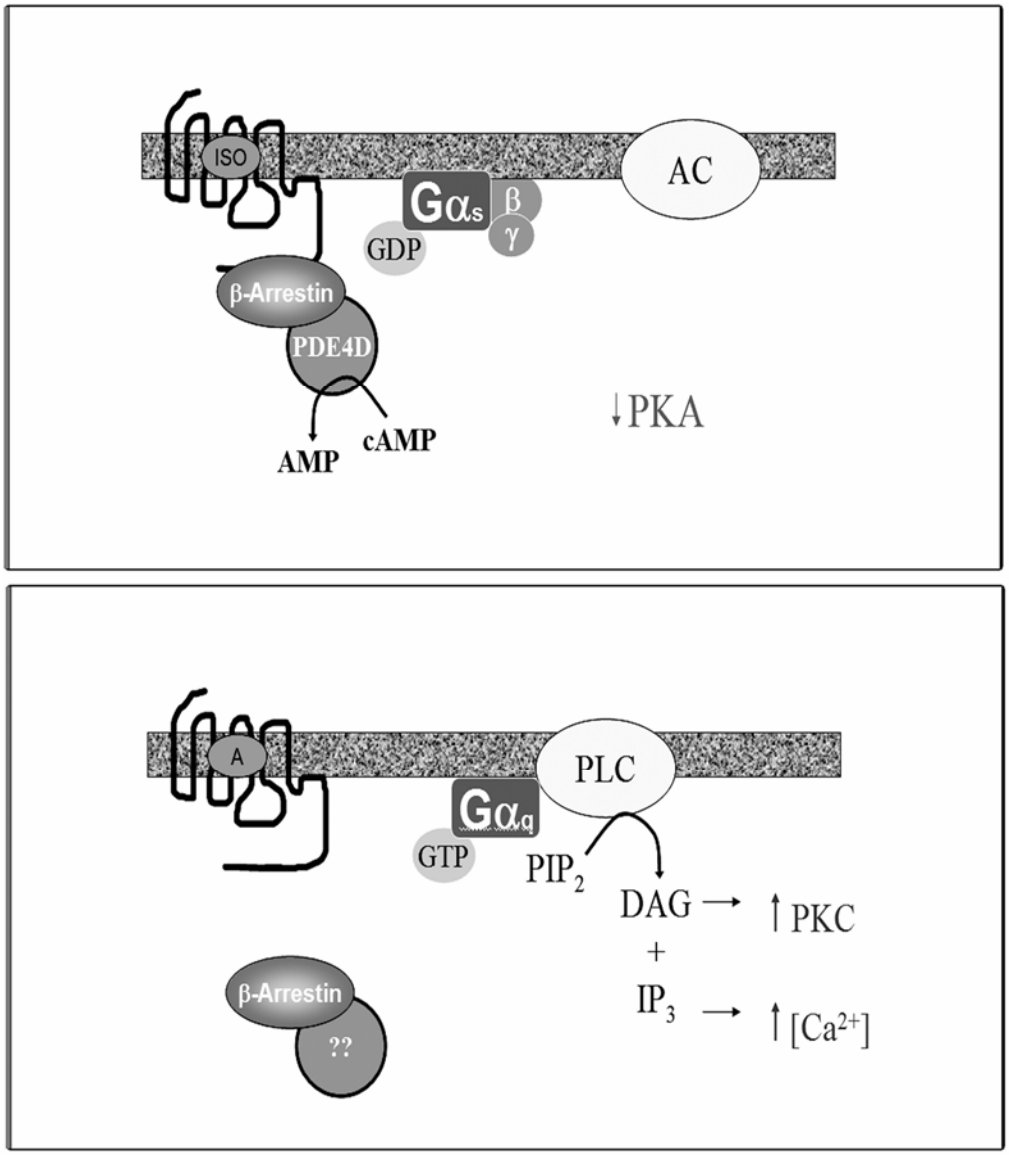


Figure 1-3: Concerted Desensitization as a General Property of β-arrestins

The central hypothesis of these studies is that a β-arrestin-DGK complex is required for degradation of the second messenger DAG in response to $G_q$-coupled receptor stimulation. This synergistically ties together the processes of receptor desensitization and degradation of DAG at the sites of PLC activity. Additionally, β-arrestin-mediated targeting of DGK could produce pools of PA at activated 7MSRs. Unlike cAMP, PA is a potent biological effector and may locally enhance PIP5K activity, increasing concentrations of $PIP_2$ which promotes both cytoskeletal rearrangement and receptor endocytosis. The specific aims of the project are as follows:

I. Develop and validate siRNA oligonucleotides for β-arrestin 1 and 2, making these valuable tools available for investigating β-arrestin-dependent signaling in primary and cultured cell types.

II. Determine if the β-arrestin-DGK complex regulates concerted desensitization for $G_q$-coupled 7TMRs and DAG, analogous to the process described for cAMP and $G_s$-coupled receptors via β-arrestin-PDE4D.

IIa. Identify the required amino acid residues governing the formation of the β-arrestin-DGK complex and the effects of β-arrestin binding on DAG kinase activity.

IIb. Investigate the roles of β-arrestin in DGK translocation to the plasma membrane and incorporation into a multi-protein complex with stimulated $G_q$-coupled 7TMRs.

III. Examine known effectors of PA for physiological signaling pathways regulated by the β-arrestin-DGK complex.

# 2. Materials and Methods

## *2.1 Materials and Reagents*

Tissue culture reagents were purchased from Invitrogen. Horseradish peroxidase-conjugated secondary antibodies and tritiated N-methyl-scopalamine were from Amersham, and Supersignal chemiluminescence reagent was from Pierce. The radiolabeled compounds [$^{125}$I]Tyr$^{4}$-angiotensin II, [$^{3}$H]cAMP, and $^{32}$P orthophosphate were purchased from Perkin Elmer. Human angiotensin II was from Peninsula Laboratories. GeneSilencer transfection reagent was bought from Gene Therapy Systems. Purified lipid standards were sold by Avanti Polar Lipids. Lipofectamine 2000 transfection reagent was obtained from Invitrogen. Thin layer chromatography plates were from Whatman International Ltd. Site-directed mutagenesis was done using a QuikChange II kit bought from Stratagene (La Jolla, CA). All other materials were purchased from Sigma.

## *2.2 DNA Plasmids*

Plasmids for the diacylglycerol kinases as well as His$_6$-Myc-tagged PIP5K Iα were graciously provided by Drs. Matt Topham and Steve Prescott at the Huntsman Cancer Institute, University of Utah. FLAG-β-arrestin 2 RRK/Q was created with sequential point mutations inserted into a FLAG-tagged rat β-arrestin plasmid. The

oligonucleotides (and their respective complimentary sequences) used in this site directed mutagenesis were (5′-3′):

R233Q- GACCGTCAAGAAGATCCAAGTGTCTGTGAGACAG

R237Q- GATCCAAGTGTCTGTGCAACAGTATGCCGAC

251Q- CGCGCGCAGTACCAGTGTCCTGTGG

The FLAG-β-arrestin 2 RRK/Q-5-kinase domain fusion protein was engineered by PCR with rat FLAG-β-arrestin 2 template and primers (5′-3′): AAATCTAGAATGGACTATAAAGACGATGACGATAAAGGTGAAAAACCCGGGA-CC and AAAAAGCTTGCAGAACTGGTCATCACCAGTCG. Cutting PCDNA 3.1 Zeo- vector and PCR product with XbaI and HinDIII and subsequent ligation produced a plasmid encoding an N-terminally FLAG-tagged β-arrestin 2 lacking a stop codon. The sequence was verified at this point by ABI sequencing at the Duke University DNA Analysis Facility. The core kinase domain of PIP5K Iα (residues 59-438) was created by PCR with primers (5′-3′): AAAAAGCTTGGTGGCGATCCCGCGGTCCCTTCC and AAAAAGCTTCTAAACAAACCTGTAAGACTG, cut with HinDIII and inserted into the FLAG-β-arrestin 2 RRK/Q plasmid. Sequencing confirmed the finished plasmid encoded FLAG-β-arrestin 2 RRK/Q open reading frame followed by two glycine residues and the 5-kinase domain with 3′ stop codon. All other plasmids used were created previously in the Lefkowitz lab or engineered as described in this text.

## *2.3 Techniques*

**Synthesis of siRNAs.** Chemically synthesized, double-stranded siRNAs, with 19-nucleotidet duplex RNA and 2-nt 3′ dTdT overhangs were purchased from Dharmacon Research or Xeragon Inc. in deprotected and desalted form. To design β-arrestin-specific siRNA duplexes, the mRNA sequences for human β-arrestin 1 and 2 were screened for unique 21-nt sequences in the NCBI database using the BLAST search algorithm [111]. The accession numbers in brackets given below are from GeneBank. The original siRNA sequences targeting β-arrestin 1 (NM_020251) and β-arrestin 2 (NM_004313) are 5′-AAAGCCUUCUGCGCGGAGAAU-3′ and 5′-AAGGACCGCAAAGUGUUUGUG-3′ and correspond to the positions 439-459 and 201-221 relative to the start codon respectively (Table 2-1). One small RNA duplex was synthesized and used as a control. This RNA, initially designed to target another β-arrestin 2 mRNA unique sequence (5′-AAGUGGACCCUGUAGAUGGCG-3′; position 101-120 from the start codon), has no silencing effects on β-arrestin expression. This control was subsequently replaced with a non-silencing siRNA (CTL) which does not correspond to any mammalian mRNA transcript. Simultaneous knockdown of both β-arrestin isoforms was accomplished with a 19-nucleotide sequence 5′-CCUGCGCCUUCCGCUAUGGU-3′ common to both mRNAs.

Table 2-1. Validated β-arrestin-specific siRNAs

| Oligonucleotide Name | mRNA Target Sequence | β-arrestin Isoform(s) Targeted | Nucleotides Targeted (Relative to Start Codon) |
|---|---|---|---|
| CTL | AAUUCUCCGAACGUGUCAUGT | None | n/a |
| WEM2 | AAGGACCGCAAAGUGUUUGUG | β-arrestin 2 | 101-120 |
| SA3 | CCAACCUCATTGAAUUUGA | β-arrestin 2 | 1112-1130 |
| CN1 | AAAGAGCGGAGAGUCUAUGUG | β-arrestin 1 | 145-167 |
| CN2 | AAAGCCUUCUGCGCGGAGAAU | β-arrestin 1 | 439-459 |
| RJL | CCUGCGCCUUCCGCUAUGGU | Both | 172-190 (βarr1); 175-193 (βarr2) |

**Cell Culture and Transfection.** HEK293 cells were maintained as described [112]. 40-50 % confluent cells in 100-mm plates, split at least 24 hr before transfection, were transfected with siRNA using the GeneSilencer transfection reagent (Gene Therapy Systems) according to the modified manufacturer's instructions. Briefly, 50 μl of the GeneSilencer transfection reagent was added to 300 μl MEM, while RNA mixtures containing 72 μl of 20 μM (~20 μg) RNA, 240 μl of siRNA diluent, and 180 μl MEM were prepared. Both solutions were allowed to stand 5-10 min at room temperature and mixed by inversion. Following 10-20 min incubation at room temperature, the entire transfection mixture was added to cells in a 100-mm plate containing 3-4 ml of fresh, serum-free MEM. After cells were incubated for 4 hr at 37 $^{o}$C, an additional 4-5 ml of MEM with 20 % FBS and 2 % penicillin/streptomycin were added to the plate. Following additional incubation for 48 hr, cells were divided into 12-well plates for β-arrestin immunoblotting and further experiments. For assays requiring transient receptor expression, the appropriate amounts of the plasmid encoding the selected receptor were transfected either two days after RNA treatment ($\beta_2$-AR) or simultaneously with RNA at the same time ($AT_{1A}R$) as above. All assays were performed at least three days after RNA transfection or two days after plasmid DNA transfection.

**Immunoprecipitation and Immunoblotting.** Cells were lysed in ice-cold glycerol lysis buffer with protease inhibitors as described [113]. Equivalent amounts of