Role of β-Arrestin-mediated Desensitization and Signaling in the Control of Angiotensin AT1a Receptor-stimulated Transcription*

Mi-Hye Lee, Hesham M. El-Shewy, Deirdre K. Luttrell, and Louis M. Luttrell

From the Departments of Medicine and Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425 and the Research Service of the Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina 29401

Heptahelical G protein-coupled receptors employ several mechanisms to activate the ERK1/2 cascade and control gene transcription. Previous work with the angiotensin AT1a receptor has shown that Gα11 activation leads to a rapid and transient rise in ERK1/2 activity, whereas β-arrestin binding supports sustained ERK1/2 activation by scaffolding a Raf-MEK-ERK complex associated with the internalized receptor. In this study, we compared the role of the two β-arrestin isoforms in AT1a receptor desensitization, ERK1/2 activation and transcription using selective RNA interference. In HEK293 cells, both the native AT1a receptor and a G protein-coupling deficient DRY/AAY mutant recruited β-arrestin1 and β-arrestin2 upon angiotensin binding and internalized with the receptor. In contrast, only β-arrestin2 supported protein kinase C-independent ERK1/2 activation by both the AT1a and DRY/AAY receptors. Using focused gene expression filter arrays to screen for endogenous transcriptional responses, we found that silencing β-arrestin1 or β-arrestin2 individually did not alter the response pattern but that silencing both caused a marked increase in the number of transcripts that were significantly up-regulated in response to AT1a receptor activation. The DRY/AAY receptor failed to elicit any detectable transcriptional response despite its ability to stimulate β-arrestin2-dependent ERK1/2 activation. These results indicate that the transcriptional response to AT1a receptor activation primarily reflects heterotrimeric G protein activation. Although β-arrestin1 and β-arrestin2 are functionally specialized with respect to supporting G protein-independent ERK1/2 activation, their common effect is to dampen the transcriptional response by promoting receptor desensitization.

G protein-coupled receptors (GPCRs) employ a variety of signaling mechanisms to regulate gene transcription. Second messenger-dependent protein kinases, such as cAMP-dependent protein kinase and protein kinase C (PKC), phosphorylate various transcriptional co-activators and co-repressors and drive transcription by cAMP and serum response elements. Other signals produced in conjunction with, or even independent of heterotrimeric G protein activation also transmit nuclear signals. Many GPCRs activate the Ras cascade through paracrine transactivation of receptor tyrosine kinases, such as epidermal growth factor (1, 2) and platelet-derived growth factor receptors (3, 4). Some appear to initiate tyrosine phosphorylation cascades controlling gene expression through direct interaction with nonreceptor tyrosine kinases, such as Src family and Janus kinases (5, 6). Recently, the arrestins, a family of four GPCR-binding proteins originally described for their role in the GPCR desensitization, have been found to interact with Src family kinases and to serve as scaffolds for activation of the extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase 3 mitogen-activated protein kinases (7–9). Interestingly, arrestin-dependent GPCR signaling does not appear to require heterotrimeric G protein activation, since G protein-uncoupled mutants of the AT1a angiotensin and β2 adrenergic receptors have been shown to activate ERK1/2 by recruiting arrestin (10, 11), and wild type AT1a, β2, and PTH1 parathyroid hormone receptors have been shown to initiate arrestin-dependent ERK1/2 activation in response to antagonist or inverse agonist ligands that promote arrestin recruitment in the absence of G protein activation (10, 12, 13). In this context the arrestin-bound GPCR functions as a passive scaffold, binding the components of the ERK1/2 cascade, c-Raf1, MEK1/2, and ERK1/2, localizing them to a membrane surface upon which Raf is activated and protecting phosphorylated ERK1/2 from rapid dephosphorylation by mitogen-activated protein kinase phosphatases (14). Indeed, the only essential role of the GPCR in the process appears to be to provide a ligand-regulated arrestin docking site, since GPCR-independent recruitment of arrestin to the plasma membrane is sufficient to activate ERK1/2 (15).

The abbreviations used are: GPCR, G protein-coupled receptor; AT1a, angiotensin II type 1a; DRY/AAY, D125A/R126A-mutated AT1a receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; G protein, guanine nucleotide-binding protein; GFP, green fluorescent protein; GRK, GPCR kinase; HA, influenza virus hemagglutinin; PKC, protein kinase C; RFP, red fluorescent protein; siRNA, small interfering RNA; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; LPA, lysophosphatidic acid.

2 The abbreviations used are: GPCR, G protein-coupled receptor; AT1a, angiotensin II type 1a; DRY/AAY, D125A/R126A-mutated AT1a receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; G protein, guanine nucleotide-binding protein; GFP, green fluorescent protein; GRK, GPCR kinase; HA, influenza virus hemagglutinin; PKC, protein kinase C; RFP, red fluorescent protein; siRNA, small interfering RNA; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; LPA, lysophosphatidic acid.

1 To whom correspondence should be addressed: Division of Endocrinology, Diabetes, and Medical Genetics, Dept. of Medicine, Medical University of South Carolina, 96 Jonathan Lucas St., 816 CSB, P. O. Box 250624, Charleston, SC 29425. Tel.: 843-792-2529; Fax: 843-792-4114; E-mail: luttrell@musc.edu.

2 The abbreviations used are: GPCR, G protein-coupled receptor; AT1a, angiotensin II type 1a; DRY/AAY, D125A/R126A-mutated AT1a receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; G protein, guanine nucleotide-binding protein; GFP, green fluorescent protein; GRK, GPCR kinase; HA, influenza virus hemagglutinin; PKC, protein kinase C; RFP, red fluorescent protein; siRNA, small interfering RNA; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; LPA, lysophosphatidic acid.
Currently available data suggest that arrestin binding mediates a distinct form of GPCR signaling that is not only G protein-independent but directly antagonistic to G protein signaling. Upon agonist binding, GPCR kinases (GRKs) rapidly phosphorylate specific sites within the intracellular domains or C-terminal tails of the receptor to generate high affinity arrestin binding sites (16). Arrestin binding uncouples the receptor and G protein leading to homologous desensitization of the receptor. In addition, the two non-visual arrestin isoforms, β-arrestin1 and β-arrestin2 (arrestin2 and arrestin3), contain C-terminal binding sites for clathrin and the β2-adaptin subunit of the AP-2 complex that allow them to target GPCRs for clathrin-mediated endocytosis (7). Thus, arrestin binding effectively serves to “switch” the receptor from a G protein signaling mode that transmits short-lived signals from the plasma membrane via small molecule second messengers and ion channels to a β-arrestin signaling mode that transmits a distinct set of signals that are initiated as the receptor internalizes and transits the intracellular compartment. Not surprisingly then, the kinetics of G protein and arrestin signaling differ. In the case of the AT1a receptor expressed in HEK293 cells, G protein-dependent ERK1/2 activation is mediated primarily via PKC activation and is rapid in onset but transient, with most of the signal waning within 10 min of stimulation (17). In contrast, the arrestin-dependent component of the signal becomes maximal at 10 min and persists for at least 30 min without attenuation. The spatial distribution of ERK1/2 activity also differs. PKC activation generates phosphorylated ERK1/2 that is distributed throughout the cytosol and nucleus, whereas the arrestin pathway activates a discrete pool of the kinase that colocalizes with the receptor-arrestin complex, initially on the plasma membrane and later in endosomal vesicles (18).

Although we are beginning to understand the mechanisms and the temporal and spatial characteristics of arrestin-mediated signals, currently little is known about the functional relevance of these and other G protein-independent signals. Some recent data suggest an important role for G protein-independent signaling in the control of gene transcription. Cardiac-specific overexpression of a G protein-uncoupled mutant AT1a receptor produces greater angiotensin-stimulated Src activation and cytosolic accumulation of active ERK1/2 and promotes a histologically distinct form of cardiac hypertrophy with greater cardiomyocyte hypertrophy and less cardiac fibrosis than that caused by a similar level of overexpression of the wild type receptor, suggesting that G protein-independent signals orchestrate a distinct subset of AT1a receptor responses (19). In response to stimulation of the δ-opioid receptor, β-arrestin1 reportedly translocates into the nucleus, where it interacts with the p27 and c-Fos promoters and stimulates transcription by recruiting histone acetyltransferase p300 and enhancing local histone H4 acetylation (20, 21). To begin to address the broad question of how β-arrestin-dependent GPCR densensitization and signaling affect transcription, we have employed selective RNA interference to determine whether silencing β-arrestin1 and/or β-arrestin2 expression affects AT1a receptor-mediated transcription and tested whether a G protein-uncoupled mutant of the AT1a receptor that mediates β-arrestin2-dependent ERK1/2 activation is able to elicit a transcriptional response. We find that both β-arrestin isoforms contribute to AT1a receptor desensitization but that only β-arrestin2 supports G protein-independent ERK1/2 activation. Analysis of the short term transcriptional response to angiotensin assayed using cDNA arrays containing markers for 96 signal-regulated transcripts revealed that silencing either β-arrestin1 or β-arrestin2 alone does not affect the transcriptional response, whereas silencing both leads to an increase in the number of AT1a receptor-regulated genes. Consistent with this, β-arrestin2-dependent ERK1/2 activation does not elicit a detectable response, even among ERK1/2-regulated genes. Our data indicate that although β-arrestin1 and β-arrestin2 are functionally specialized with respect to their roles in receptor desensitization and G protein-independent ERK1/2 activation, the transcriptional response to AT1a receptor activation primarily results from G protein-dependent signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Angiotensin II was purchased from Sigma Co. PD98059 and Ro31–8425 were from Calbiochem-EMD Biosciences Inc. Double-stranded siRNAs were purchased from Qiagen (Germantown, MD). GeneSilencer™ transfection reagent was from Gene Therapy Systems (San Diego, CA). Trizol reagent, sheared salmon sperm DNA, cell culture media, and supplements were from Invitrogen. RNase inhibitor and Moloney murine leukemia virus reverse transcriptase were from Promega Corp. (Madison, WI). FuGENE 6, biotin-16-dUTP, and High Pure RNA isolation kits were from Roche Diagnostics. SuperArray™ Human Signal Transduction PathwayFinder cDNA GEArrays were from SuperArray Bioscience Corp. (Frederick, MD). iScript™ cDNA synthesis kits and iQ SYBR Green Supermix were from Bio-Rad. FLIPRTM Calcium 3 assay kits were from Molecular Devices (Sunnyvale, CA). Polyclonal phosphorylation state-specific anti-ERK1/2 IgG was from Cell Signaling Technology Inc. (Beverly, MA), and total anti-ERK1/2 IgG was from Upstate Biotechnology Inc (Waltham, MA). Rabbit polyclonal anti-β-arrestin was a gift from Robert J. Lefkowitz (Duke University, Durham, NC). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was from Jackson Immuno Research Laboratories Inc. (West Grove, PA).

**cDNA Constructs**—The pcDNA3.1 expression plasmids encoding hemagglutinin (HA) epitope-tagged rat AT1a receptor and green fluorescent protein (GFP)-tagged β-arrestin1 and β-arrestin2 (22, 23) were gifts from Marc G. Caron (Duke University, Durham, NC). The pDsRed1-N1 expression plasmid encoding red fluorescent protein (RFP)-tagged ERK2 was prepared as described (18). The pcDNA3.1 expression plasmid encoding the D125A/R126A (DRY/AAY) AT1a receptor (24) was a gift from Laslo Hunyady (Semmelweis University, Budapest, Hungary).

**Cell Culture and Transfection**—HEK293 cells were obtained from the American Type Culture Collection and maintained in minimum essential medium with Earle’s salts supplemented with 10% fetal bovine serum and penicillin/streptomycin. Routine transient transfection of HEK293 cells with plasmids encoding wild type and DRY/AAY AT1a receptors was performed in 100-mm dishes using FuGENE 6 according to the manufacturer’s instructions with 3–6 μg of total plasmid

---

**Materials**—Angiotensin II was purchased from Sigma Co. PD98059 and Ro31–8425 were from Calbiochem-EMD Biosciences Inc. Double-stranded siRNAs were purchased from Qiagen (Germantown, MD). GeneSilencer™ transfection reagent was from Gene Therapy Systems (San Diego, CA). Trizol reagent, sheared salmon sperm DNA, cell culture media, and supplements were from Invitrogen. RNase inhibitor and Moloney murine leukemia virus reverse transcriptase were from Promega Corp. (Madison, WI). FuGENE 6, biotin-16-dUTP, and High Pure RNA isolation kits were from Roche Diagnostics. SuperArray™ Human Signal Transduction PathwayFinder cDNA GEArrays were from SuperArray Bioscience Corp. (Frederick, MD). iScript™ cDNA synthesis kits and iQ SYBR Green Supermix were from Bio-Rad. FLIPRTM Calcium 3 assay kits were from Molecular Devices (Sunnyvale, CA). Polyclonal phosphorylation state-specific anti-ERK1/2 IgG was from Cell Signaling Technology Inc. (Beverly, MA), and total anti-ERK1/2 IgG was from Upstate Biotechnology Inc (Waltham, MA). Rabbit polyclonal anti-β-arrestin was a gift from Robert J. Lefkowitz (Duke University, Durham, NC). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was from Jackson Immuno Research Laboratories Inc. (West Grove, PA).

**cDNA Constructs**—The pcDNA3.1 expression plasmids encoding hemagglutinin (HA) epitope-tagged rat AT1a receptor and green fluorescent protein (GFP)-tagged β-arrestin1 and β-arrestin2 (22, 23) were gifts from Marc G. Caron (Duke University, Durham, NC). The pDsRed1-N1 expression plasmid encoding red fluorescent protein (RFP)-tagged ERK2 was prepared as described (18). The pcDNA3.1 expression plasmid encoding the D125A/R126A (DRY/AAY) AT1a receptor (24) was a gift from Laslo Hunyady (Semmelweis University, Budapest, Hungary).

**Cell Culture and Transfection**—HEK293 cells were obtained from the American Type Culture Collection and maintained in minimum essential medium with Earle’s salts supplemented with 10% fetal bovine serum and penicillin/streptomycin. Routine transient transfection of HEK293 cells with plasmids encoding wild type and DRY/AAY AT1a receptors was performed in 100-mm dishes using FuGENE 6 according to the manufacturer’s instructions with 3–6 μg of total plasmid
**β-Arrestins and AT1α Receptor Transcription**

DNA/dish and 3 μL of FuGENE/μg of DNA. Before each experiment, transfected cells were seeded into multiwell plates as appropriate, and monolayers were incubated for 24 h in serum-free growth medium supplemented with 10 mM HEPES (pH 7.4), 0.1% bovine serum albumin, and penicillin/streptomycin. Levels of receptor expression were 0.9–1.2 pmol/mg of membrane protein in all experiments.

**Silencing β-Arrestin Expression Using RNA Interference**—Isoform-selective silencing of β-arrestins employed double-stranded siRNAs with 19-nucleotide duplex RNA and 2-nucleotide 3′ dTdT overhangs. The siRNA sequences for β-arrestin1 and β-arrestin2 were 5′-AAAGCCCUUCGCGCGGAAGAU-3′ and 5′-AAGGACCGAAAGGUUUGUG-3′, corresponding to positions 439–459 and 148–168 relative to the start codon, respectively (10). The non-silencing RNA duplex 5′-AAUCUCCGAAGUGUCAGU-3′ was used for the negative control. HEK293 cells at 40–50% confluence in 10-cm plates were transfected simultaneously with 2 μg of pcDNA3.1-HA-AT1AR or pcDNA3.1-DRY/AAY and 20 μg of control, β-arrestin1, or β-arrestin2 siRNA using 50 μL of GeneSilencer transfection reagent according to the manufacturer’s instructions. After 48 h cells were seeded into multiwell plates as appropriate and serum-deprived overnight. All assays were performed 72 h after siRNA transfection. Silencing of β-arrestin isoforms was confirmed by immunoblotting whole cell lysates of siRNA-treated cells using rabbit polyclonal anti-β-arrestin IgG with horseradish peroxidase-conjugated donkey anti-rabbit IgG as secondary antibody.

**Confocal Fluorescence Microscopy**—To visualize β-arrestin recruitment and RFP-ERK2 distribution, HEK293 cells were transfected with plasmids encoding wild type or DRY/AAY AT1α receptor, GFP-tagged β-arrestin1 or β-arrestin2, and RFP-ERK2 as specified in the figure legends. Transfected cells were seeded in collagen-coated 35-mm glass bottom dishes, stimulated as described in the figure legends, washed with Dulbecco’s phosphate-buffered saline, and fixed with 10% paraformaldehyde for 30 min at room temperature. Confocal microscopy was performed on a Zeiss LSM510 laser scanning microscope using a Zeiss 63× 1.4 numerical aperture water immersion lens using dual line switching excitation (488 nm for GFP, 568 nm for rhodamine) and emission (515–540 nm GFP, 590–610 nm rhodamine) filter sets.

**FLIPRTM Assay of Angiotensin-stimulated Calcium Influx**—Twenty-four hours after transfection, cells were passed at 70–80% confluence onto collagen-coated black well/clear bottom 96-well plates, allowed to grow for 24–48 h, then placed in serum-free medium for 5–6 h before assay. Fresh FLIPRTM Calcium 3 assay reagent (100 μL/well) was added, and plates were preincubated for an additional 1 h before stimulation. Stimulations were carried out on the FLIPRTM instrument (Molecular Devices) with 488-nm excitation wavelength per the manufacturer’s operating instructions.

**ERK1/2 Phosphorylation**—To assay ERK1/2 phosphorylation, serum-starved cell monolayers in 12-well plates were stimulated at 37 °C as described in the figure legends and lysed directly with 1× Laemmli sample buffer. Sonicated samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Phosphorylated ERK1/2 was detected by immunoblotting using rabbit polyclonal anti-phospho-ERK1/2 IgG with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Total ERK1/2, measured to confirm equal loading of whole cell lysate samples, was detected using polyclonal anti-ERK1/2 IgG. Immune complexes were visualized on x-ray film by enzyme-linked chemiluminescence and quantified using a Fluor-S Multimager.

**RNA Isolation and Gene Expression Profiling**—Appropriately transfected HEK293 cells were grown to 75% confluence in 15-cm plates and serum-starved for 16–20 h before stimulation for 90 min as described. After stimulation, cells were harvested, and total cellular RNA was isolated with Trizol reagent and purified using the RNeasy kit according to the manufacturer’s protocols. Total RNA with A260/A280 > 1.9 was used for synthesis of cDNA probes. Expression profiles were created using the SuperArrayTM Human Signal Transduction PathwayFinder cDNA GEArray. These nylon filter arrays contain 96 cDNA probes for commonly regulated transcripts along with standardization control markers. cDNA probes were synthesized from purified total RNA using GEarray-specific primers incubated with biotin-16-dUTP, Moloney murine leukemia virus reverse transcriptase, and RNase inhibitor. Gene arrays were prehybridized with sheared salmon sperm DNA before cDNA probe hybridization. Hybridization was done overnight at 60 °C for 16 h. Chemiluminescence gene array images were recorded on x-ray film. Scanalyze™ software was used to analyze optimally exposed radiographs. The abundance each transcript was normalized to housekeeping gene markers on each array.

**Gene Expression by Quantitative Real Time PCR**—The expression of Egr1 and Ptgs2 mRNA was measured by quantitative real time PCR using an iCycler iQ™ Multicolor real-time detection system. Total RNA was isolated from cells with the High Pure RNA isolation kit. cDNA for quantitative real time PCR was synthesized from 0.1 μg of RNA using the iScript™ cDNA synthesis kit amplified through one cycle by PCR with random hexamer DNA primers as templates. The specific primer sets were designed to span intron-exon borders to distinguish amplified cDNA from genomic DNA. The primers used for Egr1 amplification were: sense, 5′-GATGATGCTGT-GACAAATAAG-3′; antisense, 5′-TACGCTCAACGATTT-TAC-3′. The primers used for Ptgs2 were: sense, 5′-TTCTCTCT-TAACCTCTCTATTATAC-3′; antisense 5′-TTCCACACATC-TATTGAATC-3′. The expression level of Egr1 and Ptgs2 from each sample was normalized to the mRNA expression level of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase.

**RESULTS**

**β-Arrestin1 and β-Arrestin2 Both Support AT1α Receptor Desensitization, but Only β-Arrestin2 Mediates G Protein-independent ERK1/2 Activation**—We took two approaches to study the role of β-arrestins in modulating the transcriptional response to AT1α receptor activation. The first was to determine the effect of isoform-selective down-regulation of β-arrestin expression on wild type AT1α receptor signaling. The second was to characterize responses mediated by the G protein-uncoupled DRY/AAY mutant AT1α receptor, which we...
have previously shown activates ERK1/2 via a β-arrestin-dependent mechanism (10). Fig. 1A compares the functional G protein coupling of wild type and DRY/AAY mutant AT1a receptors, measured as intracellular calcium flux due to \( \text{G}_{\text{q,11}} \)-dependent phospholipase Cβ activation. As shown, stimulation of transiently expressed wild type AT1a receptors with angiotensin II produced a rapid and transient rise in intracellular calcium. The response was absent in untransfected HEK293 cells and cells expressing a comparable level of the DRY/AAY mutant. As shown in Fig. 1, B and C, stimulated wild type and DRY/AAY receptors internalized in complex with either GFP-tagged β-arrestin1 or β-arrestin2, confirming that both isoforms bind AT1a receptors (23, 25) and that the DRY/AAY mutant undergoes agonist-dependent sequestration despite being markedly impaired in G protein-dependent signaling (24).

Fig. 2 compares the contribution of \( \text{G}_{\text{q,11}} \)-dependent PKC activation and β-arrestin coupling to ERK1/2 activation by the wild type and DRY/AAY receptors. Endogenous β-arrestin1 and β-arrestin2 were down-regulated using isoform-selective RNA interference, as shown in Fig. 2A. Fig. 2B depicts ERK1/2 activation by the wild type AT1a receptor under conditions of varying β-arrestin expression. As shown, 5 min of exposure to angiotensin II produced robust ERK1/2 phosphorylation that was not significantly inhibited in the presence of the PKC inhibitor, Ro31–8425. Silencing β-arrestin1 expression had no significant effect on either the magnitude of the signal or its PKC dependence. In contrast, β-arrestin2 silencing reduced angiotensin II-stimulated ERK1/2 phosphorylation by about 60%, consistent with its reported function as an AT1a receptor-regulated signaling scaffold (18, 26). The residual signal was significantly Ro31–8425-sensitive, suggesting that it was transmitted in large part through \( \text{G}_{\text{q,11}} \) activation. Silencing both β-arrestin isoforms simultaneously produced a modest, but significant, enhancement in angiotensin II-stimulated ERK1/2 phosphorylation that was also partially sensitive to the PKC inhibitor. These results are consistent with a model in which both β-arrestin isoforms contribute to receptor desensitization, i.e. termination of receptor-G protein coupling, but only β-arrestin2 supports ERK1/2 activation. Results obtained with the DRY/AAY mutant also support this conclusion. As shown in Fig. 2C, stimulation of the DRY/AAY mutant produces ERK1/2 activation that is sensitive to down-regulating β-arrestin2 expression but is not significantly inhibited by PKC inhibition.

The formation of stable AT1a receptor “signalsome” complexes affects the spatial distribution of ERK1/2 such that, upon endocytosis, a fraction of the ERK1/2 pool colocalizes in endosomes containing the internalized β-arrestin-bound receptor (18, 27). This is shown in Fig. 3, where the redistribution of co-transfected GFP-β-arrestin2 and RFP-ERK2 upon AT1a receptor stimulation was monitored using confocal fluorescence microscopy. In unstimulated cells, GFP-β-arrestin2 was predominantly cytosolic in distribution, whereas RFP-ERK2 was found diffusely throughout the cytosol and nucleus. After 30 min of exposure to angiotensin II, GFP-β-arrestin2 had redistributed to the endosomal pool that contains the sequestered AT1a receptor (23). At the same time a substantial
β-Arrestins and AT1a Receptor Transcription

A. Representative immunoblot (IB) depicting isoform-selective down-regulation of β-arrestin1 (βArr1) or β-arrestin2 (βArr2) expression by RNA interference. B, effect of β-arrestin1 and β-arrestin2 down-regulation on wild type AT1a receptor-mediated ERK1/2 activation. HEK293 cells were cotransfected with HA-tagged AT1a receptor plus either scrambled RNA oligonucleotide (SCR) or siRNA specific for β-arrestin1 or β-arrestin2 as indicated. Serum-deprived cells were preincubated for 30 min with or without Ro31–8425 (10 µM) before exposure for 5 min to vehicle or angiotensin II (AngII; 10 µM), and ERK1/2 phosphorylation in whole cell lysates was determined by immunoblotting.

C. Representative phospho-ERK1/2 immunoblot shows above a bar graph representing the mean ± S.E. from three independent experiments. ERK1/2 phosphorylation is expressed as the percent of maximal ERK1/2 phosphorylation observed in stimulated cells transfected with scrambled RNA oligonucleotide. *, less than SCR-treated, p < 0.05; †, greater than SCR-treated, p < 0.05; ‡, Ro31–8425-treated less than untreated, p < 0.05. C, effect of β-arrestin1 and β-arrestin2 down-regulation on DRY/AAY receptor-mediated ERK1/2 activation. HEK293 cells were cotransfected with DRY/AAY receptor plus either scrambled RNA oligonucleotide (SCR) or siRNA specific for β-arrestin2 (left panels) or preincubated for 30 min with or without Ro31–8425 (right panels) before exposure for 5 min to vehicle or angiotensin II and phospho-ERK1/2 immunoblotting. In each case a representative phospho-ERK1/2 immunoblot is shown above a bar graph representing the mean ± S.E. from three independent experiments. ERK1/2 phosphorylation is expressed as the percent of maximal ERK1/2 phosphorylation under control conditions. *, less than SCR-treated, p < 0.05; N.S., not significant.

FIGURE 2. Effect of siRNA-mediated down-regulation of β-arrestin1 and β-arrestin2 expression on ERK1/2 activation by wild type AT1a and DRY/AAY receptors. A, representative immunoblot (IB) depicting isoform-selective down-regulation of β-arrestin1 (βArr1) or β-arrestin2 (βArr2) expression by RNA interference. B, effect of β-arrestin1 and β-arrestin2 down-regulation on wild type AT1a receptor-mediated ERK1/2 activation. HEK293 cells were cotransfected with HA-tagged AT1a receptor plus either scrambled RNA oligonucleotide (SCR) or siRNA specific for β-arrestin1 or β-arrestin2 as indicated. Serum-deprived cells were preincubated for 30 min with or without Ro31–8425 (10 µM) before exposure for 5 min to vehicle or angiotensin II (AngII; 10 µM), and ERK1/2 phosphorylation in whole cell lysates was determined by immunoblotting. A representative phospho-ERK1/2 immunoblot is shown above a bar graph representing the mean ± S.E. from three independent experiments. ERK1/2 phosphorylation is expressed as the percent of maximal ERK1/2 phosphorylation observed in stimulated cells transfected with scrambled RNA oligonucleotide. *, less than SCR-treated, p < 0.05; †, greater than SCR-treated, p < 0.05; ‡, Ro31–8425-treated less than untreated, p < 0.05. C, effect of β-arrestin1 and β-arrestin2 down-regulation on DRY/AAY receptor-mediated ERK1/2 activation. HEK293 cells were cotransfected with DRY/AAY receptor plus either scrambled RNA oligonucleotide (SCR) or siRNA specific for β-arrestin2 (left panels) or preincubated for 30 min with or without Ro31–8425 (right panels) before exposure for 5 min to vehicle or angiotensin II and phospho-ERK1/2 immunoblotting. In each case a representative phospho-ERK1/2 immunoblot is shown above a bar graph representing the mean ± S.E. from three independent experiments. ERK1/2 phosphorylation is expressed as the percent of maximal ERK1/2 phosphorylation under control conditions. *, less than SCR-treated, p < 0.05; N.S., not significant.

FIGURE 3. Effect of wild type AT1a and DRY/AAY receptor activation on the cellular distribution of β-arrestin2 and ERK1/2. HEK293 cells expressing wild type (upper panels) or DRY/AAY mutant AT1a receptors (lower panels) along with GFP-tagged β-arrestin2 (βArr2-GFP) and RFP-tagged ERK2 (RFP-ERK2) were exposed to vehicle (NS) or angiotensin II (AngII; 10 µM) for 30 min, after which cells were fixed and examined by confocal fluorescence microscopy as described. For each condition the single channel images show GFP-β-arrestin2 (green) and RFP-ERK2 (red). Colocalization of GFP-β-arrestin2 and RFP-ERK2 is shown in the overlay images (yellow). Each panel depicts a representative field of cells from one of three separate experiments that produced similar results.

β-Arrestin Expression Dampens AT1a Receptor-mediated Transcription—From the preceding, it is clear that with respect to the AT1a receptor, β-arrestin1 functions primarily in receptor desensitization and sequestration. Although it binds the receptor (23), removing β-arrestin1 has little effect on G protein coupling or ERK1/2 activation, since β-arrestin2 is sufficient to compensate for its absence (29). In contrast, β-arrestin2 performs a dual role, contributing to receptor desensitization and also supporting G protein-independent ERK1/2 activation in the context of receptor-based signal-somes. To determine the role of β-arrestins in short term nuclear signaling by the AT1a receptor, we employed gene

amount of the cytosolic pool of RFP-ERK2 redistributed such that it co-localized with GFP-β-arrestin2, whereas the nuclear pool of RFP-ERK2 was retained in the nucleus, consistent with its being tethered by binding to nuclear proteins (28). As shown, stimulus-dependent signalosome assembly is independent of G protein activation, since the DRY/AAY mutant receptor behaved similarly to the wild type.
expression filter arrays consisting of 96 marker genes representing 18 different regulatory pathways to profile the endogenous transcriptional response to angiotensin II under conditions of varying β-arrestin expression. Fig. 4A presents a representative pair of arrays generated from HEK293 cells transiently expressing AT1a receptors and transfected with control scrambled siRNA. Fig. 4B depicts the results from six replicate experiments. As shown, 90 min of stimulation with angiotensin II produced a significant increase in the transcription of five genes, two cyclin-dependent protein kinases, CDKN1A and CDKN1C, the zinc finger transcription factor early growth response 1 (Egr1), the E3-ubiquitin ligase mdm2, and cyclooxygenase 2 (Ptgs2). To validate these results and determine the contribution of PKC and ERK1/2 to the response, we performed quantitative real-time PCR on Egr1 and Ptgs2 after angiotensin stimulation in the absence or presence of Ro31–8425 or the MEK1/2 inhibitor PD98059. As shown in Fig. 5, both the Egr1 and Ptgs2 responses were partially sensitive to each inhibitor, suggesting that both PKC and ERK1/2 activity contribute to the transcriptional response.

To determine how the β-arrestin content of the cell modifies the response, we repeated the array experiments after down-regulating expression of β-arrestin1, β-arrestin2, or both using isoform-selective RNA interference. Fig. 6 shows the response profile generated under each condition, focusing only on the 10 genes for which a statistically significant response was detected under at least one condition. Although only the Ptgs2 and Egr1 responses reached statistical significance in β-arrestin1 knockdown cells and only the Ptgs2, Egr1, and CdkN1C responses were significant in β-arrestin2 knockdown cells, it is clear that down-regulating β-arrestin isoforms individually did not affect the overall pattern of mRNA abundance or the response to angiotensin II. However, a markedly different result was obtained when both β-arrestins were targeted simultaneously. First, the basal abundance of several mRNA species fell relative to the housekeeping genes used for normalization. Because basal levels of cAMP and inositol phosphates rise under these conditions (30, 31), this probably reflects compensatory down-regulation of GPCR-regulated genes due to the increased basal activity in heterotrimeric G protein signaling pathways. Second, upon stimulation, angiotensin II elicited a total of 10 significant responses; all of those seen in β-arrestin-replete cells plus six additional transcripts that were not significantly up-regulated as long as one β-arrestin isoform was present. This result, in which either β-arrestin isoform is sufficient to restrain the transcriptional response, parallels their interchangeability with respect to receptor desensitization (29). The most likely explanation is that the majority of AT1a receptor-regulated genes are controlled by heterotrimeric G protein activation and that the role of β-arrestins is to limit the duration of G protein activation by desensitizing the receptor.

G Protein-independent Signals Are Not Sufficient to Elicit Short Term Transcriptional Responses via the AT1a Receptor—If G protein-independent ERK1/2 activation via β-arrestin2 coupling was sufficient to drive transcription, we hypothesized that the DRY/AAY mutant would be capable of eliciting a transcriptional response. Thus, we generated transcription profiles
**β-Arrestins and AT1a Receptor Transcription**

**FIGURE 6.** Effect of isoform-selective down-regulation of β-arrestins on angiotensin II-stimulated mRNA transcription. HEK293 cells were cotransfected with HA-tagged AT1a receptor plus either scrambled RNA oligonucleotide or siRNA specific for β-arrestin1 (βArr1), β-arrestin2 (βArr2), or both (βArr1/2) as indicated. A, representative immunoblot (IB) depicting β-arrestin1 or β-arrestin2 down-regulation by RNA interference. B, cells were exposed to vehicle or angiotensin II (AngII; 10 μM) for 90 min before RNA isolation and gene expression profiling using SuperArray™ Human Signal Transduction PathwayFinder cDNA GEArrays as described. Each bar graph depicts the relative mRNA abundance in vehicle and angiotensin II-treated cells for the 10 mRNA species that showed a significant increase under at least one condition. Data shown represent the mean ± S.E. in three separate experiments. *p < 0.05.

**FIGURE 7.** Gene expression profiling of HEK293 cells expressing DRY/AAY mutant receptors. HEK293 cells were cotransfected with DRY/AAY receptor plus either scrambled RNA oligonucleotide or siRNA specific for β-arrestin2 (βArr2) as indicated. A, representative immunoblot (IB) depicting β-arrestin2 down-regulation by RNA interference. B, serum-deprived cells were exposed to vehicle or angiotensin II (AngII; 10 μM) for 90 min before RNA isolation and gene expression profiling using SuperArray™ Human Signal Transduction PathwayFinder cDNA GEArrays as described. The inset immunoblots depict representative time courses of ERK1/2 phosphorylation (P-ERK1/2) in cells stimulated from 0 to 120 min with angiotensin II. Each bar graph depicts the relative mRNA abundance in vehicle and angiotensin II-treated cells for the 10 mRNA species shown previously for wild type AT1a receptor-expressing cells. Data shown represent mean ± S.E. values in three separate experiments. C, HEK293 cells were cotransfected with empty vector or DRY/AAY receptor as indicated. Unstimulated and angiotensin II stimulated Egr1 and Ptgs2 mRNA abundance was determined by quantitative real-time PCR as described. Data shown represent the mean ± S.E. values in three separate experiments.

from β-arrestin-replete DRY/AAY-expressing HEK293 cells and cells in which β-arrestin2 expression was down-regulated. As shown in Fig. 7, exposure to angiotensin II did not elicit a significant response in any of the ten AT1a receptor-responsive genes previously identified despite producing a sustained increase in ERK1/2 phosphorylation. Nor were significant
responses detected in any of the other genes represented on the array (data not shown). Down-regulating β-arrestin2 expression blocked DRY/AAY-mediated ERK1/2 activation but did not change mRNA abundance in either stimulated or unstimulated cells. The failure of the DRY/AAY receptor to promote Egr1 or Ptgs2 transcription was confirmed by quantitative real time PCR. As with results obtained using the wild type AT1a receptor, these results suggest that most if not all of the short term transcriptional response to AT1a receptoractivation in this system requires G protein activation.

DISCUSSION

In this study we compared the role of the two β-arrestin isoforms in AT1a receptor desensitization, ERK1/2 activation and transcriptional regulation. Our results demonstrate that β-arrestin1 and β-arrestin2 perform both shared and unique functions. Both isoforms form stable complexes with the receptor and contribute to receptor desensitization and sequestration, whereas only β-arrestin2 supports G protein-independent ERK1/2 activation. When viewed from the perspective of nuclear signaling, the two isoforms again appear similar. Silencing β-arrestin1 or β-arrestin2 expression individually had no real qualitative or quantitative impact on the pattern of angiotensin-stimulated transcription surveyed using cDNA arrays representing 96 different signal-regulated genes. Thus, loss of β-arrestin2-dependent ERK1/2 activation had no detectable effect on nuclear signaling by the AT1a receptor. Consistent with this, stimulation of the DRY/AAY receptor mutant failed to elicit transcription of even ERK1/2-regulated genes such as Egr1 and Ptgs2. Simultaneous down-regulation both β-arrestins increased the number of transcripts that were significantly expressed but did not qualitatively change the pattern of gene expression. These results support the hypothesis that transcriptional responses to AT1a receptor activation primarily reflect heterotrimeric G protein signaling, including G protein-dependent ERK1/2 activation, whereas ERK1/2 activated via the β-arrestin2 pathway does not affect transcription.

Although it is clear that β-arrestins play a role in the generation of a subset of GPCR signals, very little is known about the physiologic relevance of β-arrestin signaling. In contrast even to heterotrimeric G proteins, where there are 16 known mammalian Gα subunit genes, five Gβ subunit genes, and 12 Gγ subunit genes (32), there are only two genes encoding β-arrestins, they are ubiquitously expressed outside the retina, and they bind to the vast majority of GPCRs (7). This suggests that β-arrestin signaling should be rather “bland”; i.e. it should carry out relatively few functions, and those should be fundamental to GPCR signaling, such as regulation of receptor endocytosis and trafficking. On the other hand, some indirect evidence suggests that β-arrestin-mediated signaling may be more diverse. First, the pattern of β-arrestin binding is not linked to the G protein-coupling specificity of the receptor. Receptors such as the Gs-coupled β2 adrenergic and D1A dopamine, the Gq/11-coupled α1B adrenergic, and the Gα12,13-coupled lysophosphatidic acid (LPA) and μ opioid preferentially bind to β-arrestin 2. For these receptors, the β-arrestin interaction is transient, i.e. the receptor-β-arrestin complex dissociates as the receptor internalizes. In contrast, the Gs-coupled V2 vasopressin and Gq/11-coupled AT1a receptors recruit both β-arrestin 1 and β-arrestin 2 and form stable receptor-arrestin complexes that internalize as a unit that is targeted to endosomes (23). Although receptors of both types appear to employ β-arrestins to generate sustained ERK1/2 activity (11, 17), the subsequent targeting of ERK1/2 differs between them. In the case of the AT1a, NK1 neurokinin, and PAR2 receptors, the active kinase clearly remains associated with a receptor-based signalosome that localizes to endosomes (18, 33, 34). In contrast, we have found that ERK1/2 activated by the LPA receptor in an apparently β-arrestin-dependent manner is able to translocate to the cell nucleus (35). Second, β-arrestins appear to adopt different conformations depending on which GPCR they bind and which GRK phosphorylated the receptor. Evidence of the former comes from characterization of β-arrestin2 ubiquitination. Upon binding the AT1a receptor, lysines 11 and 12 of β-arrestin2 are ubiquitinated. A β-arrestin2 (K11R,K12R) mutant that cannot be ubiquitinated does not remain stably bound to the AT1a receptor and cannot support signals and assembly. However, this same mutant is ubiquitinated and remains fully functional when recruited to the V2 receptor (27). The variable pattern of ubiquitination suggests either that the conformation or the accessibility of surface epitopes of β-arrestin must differ depending on the GPCR binding partner. Similarly, experiments using RNA interference to target specific GRK isoforms have shown that GRK2 and GRK3 phosphorylation of the V2 receptor promotes β-arrestin-dependent desensitization, whereas GRK5 and GRK6 phosphorylation appears to be exclusively responsible for initiating β-arrestin-dependent ERK1/2 activation (36). The physical basis for this specialization remains unclear, but it may indicate that phosphorylation of specific GRK sites on the GPCR preferentially induces different desensitizing and signaling conformations of β-arrestin2. The third factor is the relatively large number of signaling proteins that have been reported to bind β-arrestins, among them Src family kinases, Raf/MEK/ERK, Ask1/MK4/c-Jun N-terminal kinase 3, the E3 ubiquitin ligase, mdm2, the cAMP phosphodiesterases PDE4D3/5, and the Dishevelled proteins Dvl 1 and Dvl 2 (8, 9). Whether or not all of these putative binding partners produce physiologically relevant signals, it is unlikely that they all interact with β-arrestin-bound GPCRs at the same time and in the same cells. Although a few of these proteins, e.g. c-Jun N-terminal kinase 3 (37, 38), show a clear binding preference for a specific β-arrestin isoform, others, e.g. ERK1/2 (18, 34), interact with both. Nonetheless, selective siRNA knockdown of the two β-arrestins suggest that β-arrestin2 mediates AT1a receptor-stimulated ERK1/2 activation, whereas β-arrestin1 does not (17).

Collectively, these data suggest that β-arrestins affect GPCR desensitization, trafficking and signaling in proportions that vary depending on the receptor, the GRK, and the cellular context in which they occur. In examining the contribution of endogenous β-arrestins to transcriptional activation by the AT1a receptor, we have studied one such context. Previous work on the effect of β-arrestins on ERK1/2-dependent transduction focused on β-arrestin overexpression and used Elk1-luciferase reporters to detect nuclear ERK1/2 signaling (18, 39, 30).
The experiments clearly showed that overexpressed β-arrestin increases total cellular ERK1/2 phosphorylation but inhibits nuclear translocation and Elk1-dependent transcription by retaining ERK1/2 in cytosolic receptor-arrestin complexes. Such results are predictable given that β-arrestins are in effect cytosolic mitogen-activated protein kinase-binding proteins, particularly β-arrestin2, which possesses a discrete nuclear export sequence (41, 42). Similar effects have been reported in cells overexpressing a catalytically inactive mutant of mitogen-activated protein (MAP) kinase phosphatase 2 (43), another cytosolic MAP kinase binding protein but one that is clearly not involved in physiologic ERK1/2 activation. Thus, we chose in the present study to employ a loss of function approach to determine the role of endogenous β-arrestin isoforms in the transcription of endogenous AT1a receptor-regulated genes. Using this more physiologic approach we find that attenuating β-arrestin-dependent desensitization enhances angiotensin-stimulated transcription, whereas the loss of β-arrestin2-mediated ERK1/2 activation does not affect the response. Similarly, activation of β-arrestin2-mediated ERK1/2 activation by the G protein-uncoupled DRY/AAY receptor fails to elicit a nuclear response. These data support the hypothesis that G protein signaling accounts for most, if not all, of the short term transcriptional response to AT1a receptor activation in HEK293 cells.

Our results with the AT1a receptor contrast with results we obtained studying LPA-stimulated transcription in murine embryon fibroblasts from β-arrestin1/2 null embryos (35) and point out that the role of β-arrestin-dependent ERK1/2 activation in nuclear signaling may differ betweenGPCRs. Consistent with the present report, we found that reintroducing β-arrestin2 into the β-arrestin1/2 null background conferred sustained G protein-independent ERK1/2 activation but reduced the number of endogenous genes that were up-regulated by LPA. Surprisingly, however, we found that the LPA-stimulated responses that remained were ERK1/2-dependent despite the apparent β-arrestin dependence of the ERK1/2 activation. We speculate that the difference relates to the pattern of β-arrestin binding exhibited by the receptors. Unlike the AT1a receptor, LPA receptors form transient receptor-arrestin complexes. If, as appears likely, β-arrestin2 still functions as an ERK1/2 activation scaffold for the LPA receptor, then dissociation of the complex upon internalization may permit the ERK1/2 to gain access to the nuclear compartment and drive transcription. Consistent with this hypothesis, we have found that exchanging the C terminus of the V2 receptor for that of the β2 adrenergic receptor, which changes the V2 receptor-arrestin interaction from stable to transient, confers upon the chimera the ability to stimulate an Elk1-luciferase reporter (40).

Presumably, the existence of distinct mechanisms of ERK1/2 activation allows GPCRs to control the substrate specificity and function of these multifunctional kinases. In addition to phosphorylating nuclear transcription factors, ERK1/2 targets numerous plasma membrane, cytoplasmic, and cytoskeletal substrates (28). Some data suggest that β-arrestins may regulate receptor desensitization, trafficking, and chemotactic cell migration in part by directing ERK1/2 toward non-nuclear substrates. Several proteins that modulate GPCR signaling are ERK1/2 substrates, including β-arrestin 1 (44), GRK2 (45, 46), and the Go subunit-interacting protein GAIP (47). Thus, one role of β-arrestin-bound ERK1/2 might be to enhance the efficiency of phosphorylation of proteins involved in GPCR regulation. Another possible role is in the control of chemotactic cell migration. T and B cells from β-arrestin2 knock-out mice exhibit strikingly impaired chemotaxis in transwell and transendothelial migration assays (48). In NIH-3T3 cells, PAR2 receptors stimulate prolonged activation of ERK1/2 in receptor-β-arrestin-ERK1/2 complexes. In a chemotactic gradient, these complexes are enriched in pseudopodia. PAR-2 receptor-mediated cytoskeletal reorganization, polarized pseudopod extension, and chemotaxis are ERK1/2-dependent and inhibited by expression of a dominant negative mutant of β-arrestin 1, suggesting that the formation of β-arrestin-ERK1/2 signaling complexes at the leading edge of a cell directs localized actin assembly and drives chemotaxis (49). Although such data suggest that the ultimate function of GPCR-activated ERK1/2 is determined by its mechanism of activation, a thorough understanding of how G protein and β-arrestin signals are integrated to affect cell function will require further investigation using different GPCRs expressed in a variety of cell types.

REFERENCES

1. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) Nature 402, 884–888
2. Daub, H., Wallash, C., Lankenau, A., Herrlich, A., and Ullrich, A. (1997) EMBO J. 16, 7032–7044
3. Heenan, S., Haendeler, J., Saito, Y., Ishida, M., and Berk, B. C. (2000) J. Biol. Chem. 275, 15926–15932
4. Linseman, D. A., Benjamin, C. W., and Jones, D. A. (1995) J. Biol. Chem. 270, 12563–12568
5. Ali, M. S., Sayeski, P. P., Dirksen, L. B., Hayzer, D. J., Marrero, M. B., and Bernstein, K. E. (1997) J. Biol. Chem. 272, 23382–23388
6. Marrero, M. B., Venema, V. J., Ju, H., Eaton, D. C., and Venema, R. C. (1998) Am. J. Physiol. 275, C1216–C1223
7. Ferguson, S. S. (2001) Pharmacol. Rev. 53, 1–24
8. Luttrell, L. M. (2005) J. Mol. Neurosci. 26, 253–264
9. Shenoy, S. K., and Lefkowitz, R. J. (2005) Sci. STKE 2005, cm10
10. Wei, H., Ahn, S., Shenoy, S. K., Karnik, S., Hunyady, L., Luttrell, L. M., and Lefkowitz, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 10782–10787
11. Shenoy, S. K., Drake, M. T., Nelson, C. D., Houz, D. A., Xiao, K., Madabushi, S., Reiter, E., Premont, R. T., Lichtarge, O., and Lefkowitz, R. J. (2006) J. Biol. Chem. 281, 1261–1273
12. Azzi, M., Charest, P. G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M., and Pineyro, G. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 11406–11411
13. Gesty-Palmer, D., Chen, M., Reiter, E., Ahn, S., Nelson, C. D., Wang, S., Eckhardt, A. E., Cowan, C. L., Spurney, R. F., Luttrell, L. M., and Lefkowitz, R. J. (2006) J. Biol. Chem. 281, 1086–10864
14. Jafari, E., El-Shewy, H. M., Lee, M.-H., Kelly, M., Luttrell, D. K., and Luttrell, L. M. (2006) J. Biol. Chem. 281, 19346–19357
15. Terrillon, S., and Bouvier, M. (2004) EMBO J. 23, 3950–3961
16. Stoffel, R. H., III, Pitcher, J. A., and Lefkowitz, R. J. (1997) J. Membr. Biol. 157, 1–8
17. Ahn, S., Shenoy, S. K., Wei, H., and Lefkowitz, R. J. (2004) J. Biol. Chem. 279, 35518–35525
18. Luttrell, L. M., Roubadous, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2449–2454
19. Zhai, P., Yamamoto, M., Galeotti, J., Liu, J., Masurekar, M., Thaisz, J., Irie, K., Holle, E., Yu, X., Kupershmidt, S., Roden, D. M., Wagner, T., Yatani, A., Vatner, D. E., Vatner, S. F., and Sadoshima, J. (2005) J. Clin. Investig. 115, 3045–3056
20. Kang, J., Shi, Y., Xiang, B., Qu, B., Su, W., Zhu, M., Zhang, M., Bao, G., Wang, F., Zhang, X., Yang, R., Fan, F., Chen, X., Pei, G., and Ma, L. (2005)
Cell 123, 833–847

21. Ma, L., and Pei, G. (2007) J. Cell Sci. 120, 213–218
22. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (1999) J. Biol. Chem. 274, 32248–32257
23. Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G., and Barak, L. S. (2000) J. Biol. Chem. 275, 17201–17210
24. Gaborik, Z., Jagadeesh, G., Zhang, M., Spat, A., Catt, K. J., and Hunyady, L. (2003) Endocrinology 144, 2220–2228
25. Zhang, J., Barak, L. S., Anborgh, P. H., Laporte, S. A., Caron, M. G., and Ferguson, S. S. (1999) J. Biol. Chem. 274, 10999–11006
26. Ahn, S., Wei, H., Garrison, T. R., and Lefkowitz, R. J. (2004) J. Biol. Chem. 279, 7807–7811
27. Shenoy, S. K., and Lefkowitz, R. J. (2005) J. Biol. Chem. 280, 15315–15324
28. Pearson, G., Robinson, F., Beers Gibosn, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001) Endocr. Rev. 22, 153–183
29. Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1601–1606
30. Ahn, S., Nelson, C. D., Garrison, T. R., Miller, W. E., and Lefkowitz, R. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1740–1744
31. Wei, H., Ahn, S., Barnes, W. G., and Lefkowitz, R. J. (2004) J. Biol. Chem. 279, 48255–48261
32. Downes, G. B., and Gautam, N. (1999) Genomics 62, 544–552
33. DeFea, K. A., Vaughan, Z. D., O'Bryan, E. M., Nishijima, D., Dery, O., and Bunnett, N. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11086–11091
34. DeFea, K. A., Zalesky, J., Thoma, M. S., Dery, O., Mullins, R. D., and Bunnett, N. W. (2000) J. Cell Biol. 148, 1267–1281
35. Gesty-Palmer, D., El-Shewy, H., Kohout, T. A., and Luttrell, L. M. (2005) J. Biol. Chem. 280, 32157–32167
36. Ren, X. R., Reiter, E., Ahn, S., Kim, J., Chen, W., and Lefkowitz, R. J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1448–1453
37. McDonald, P. H., Chow, C.-W., Miller, W. E., LaPorte, S. A., Field, M. E., Lin, F.-T., Davis, R. I., and Lefkowitz, R. J. (2000) Science 290, 1574–1577
38. Miller, W. E., McDonald, P. H., Cai, S. F., Field, M. F., Davis, R. J., and Lefkowitz, R. J. (2001) J. Biol. Chem. 276, 27770–27777
39. Tohgo, A., Pierce, K. L., Choy, E. W., Lefkowitz, R. J., and Luttrell, L. M. (2002) J. Biol. Chem. 277, 9429–9436
40. Tohgo, A., Choy, E. W., Gesty-Palmer, D., Pierce, K. L., Laporte, S., Oakley, R., Caron, M. G., Lefkowitz, R. J., and Luttrell, L. M. (2003) J. Biol. Chem. 278, 6258–6267
41. Scott, M. G., Le Rouzic, E., Perianin, A., Pierotti, V., Enslen, H., Benichou, S., Marullo, S., and Benmerah, A. (2002) J. Biol. Chem. 277, 37693–37701
42. Wang, P., Wu, Y., Ge, X., Ma, L., and Pei, G. (2003) J. Biol. Chem. 278, 11648–11653
43. Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S., and Pouyssegur, J. (1999) EMBO J. 18, 664–674
44. Lin, F.-T., Miller, W. E., Luttrell, L. M., and Lefkowitz, R. J. (1999) J. Biol. Chem. 274, 15971–15974
45. Pitcher, J. A., Tesmer, J. J., Freeman, J. L., Capel, W. D., Stone, W. C., and Lefkowitz, R. J. (1999) J. Biol. Chem. 274, 34531–34534
46. Elorza, A., Sarnago, S., and Mayor, F., Jr. (2000) Mol. Pharmacol. 57, 778–783
47. Ogier-Denis, E., Pattingre, S., El Benna, J., and Codogno, P. (2000) J. Biol. Chem. 275, 39090–39095
48. Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., and Patel, D. D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7478–7483
49. Ge, L., Ly, Y., Hollenberg, M., and DeFea, K. (2003) J. Biol. Chem. 278, 34418–34426