β-Arrestin Scaffolding of the ERK Cascade Enhances Cytosolic ERK Activity but Inhibits ERK-mediated Transcription following Angiotensin AT1a Receptor Stimulation*

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Akira Tohgo‡§§, Kristen L. Pierce‡§§, Eric W. Choy¶¶, Robert J. Lefkowitz¶¶¶** and Louis M. Luttrell¶¶¶¶§§§

From the ‡Howard Hughes Medical Institute and the Departments of ¶¶¶¶¶ Medicine and ¶¶¶¶¶¶¶¶¶¶¶¶ Biochemistry, Duke University Medical Center, Durham, North Carolina 27710 and the §§Geriatics Research, Éducation, and Clinical Center, Durham Veterans Affairs Medical Center, Durham, North Carolina 27705

β-Arrestins are cytosolic proteins that mediate homologous desensitization of G protein-coupled receptors (GPCRs) by binding to agonist-occupied receptors and by uncoupling them from heterotrimeric G proteins. The recent finding that β-arrestins bind to some mitogen-activated protein (MAP) kinases has suggested that they might also function as scaffolds for GPCR-stimulated MAP kinase activation. To define the role of β-arrestins in the regulation of ERK MAP kinases, we examined the effect of β-arrestin overexpression on ERK1/2 activation and nuclear signaling in COS-7 cells expressing angiotensin II type 1a receptors (AT1aRs). Expression of either β-arrestin1 or β-arrestin2 reduced angiotensin-stimulated phosphatidylinositol hydrolysis but paradoxically increased angiotensin-stimulated ERK1/2 phosphorylation. The increase in ERK1/2 phosphorylation in β-arrestin-expressing cells correlated with activation of a β-arrestin-bound pool of ERK2. The β-arrestin-dependent increase in ERK1/2 phosphorylation was accompanied by a significant reduction in ERK1/2-mediated, Elk1-driven transcription of a luciferase reporter. Analysis of the cellular distribution of phospho-ERK1/2 by confocal immunofluorescence microscopy and cellular fractionation revealed that overexpression of β-arrestin resulted in a significant increase in the cytosolic pool of phospho-ERK1/2 and a corresponding decrease in the nuclear pool of phospho-ERK1/2 following angiotensin stimulation. β-Arrestin overexpression resulted in formation of a cytoplasmic pool of β-arrestin-bound phospho-ERK, decreased nuclear translocation of phospho-ERK1/2, and inhibition of Elk1-driven luciferase transcription even when ERK1/2 was activated by overexpression of cRaf-1 in the absence of AT1aR stimulation. These data demonstrate that β-arrestins facilitate GPCR-mediated ERK activation but inhibit ERK-dependent transcription by binding to phospho-ERK1/2, leading to its retention in the cytosol.

The G protein-coupled receptor (GPCR)1 superfamily is composed of a diverse array of membrane receptors that share a conserved seven-transmembrane domain architecture. In response to receptor occupancy, GPCRs promote the activation of heterotrimeric G proteins by catalyzing the exchange of GDP for GTP on the Gα subunit and dissociation of the Gα subunit from the Gβγ subunit heterodimer. Once dissociated, free Gα-GTP and Gβγ subunits regulate the activity of enzymatic effectors, such as adenyl cyclases and phospholipase C isoforms.

For the majority of GPCRs, productive G protein coupling in the continued presence of agonist is terminated by receptor phosphorylation followed by the binding of arrestins (1, 2). Specialized G protein-coupled receptor kinases phosphorylate agonist-occupied GPCRs, increasing their affinity for arrestins. Upon binding the receptor, arrestins sterically block further coupling between GPCR and G protein. In addition, the two non-visual arrestins, β-arrestin1 and 2, target GPCRs for endocytosis by linking the GPCR to components of the cellular endocytic machinery, including clathrin and AP-2 (3, 4).

Besides their well characterized roles in GPCR desensitization and sequestration, recent evidence suggests that β-arrestins may also contribute to GPCR signaling by functioning as adaptors or scaffolds for the recruitment of signaling molecules into complex with agonist-occupied receptors. By binding to both the non-receptor tyrosine kinase, c-Src, and to agonist-occupied β-adrenergic receptors, β-arrestin1 can confer tyrosine kinase activity upon the receptor (5). Similarly, β-arrestins are involved in recruiting c-Src to the neurokinin-1 receptor in KNRR kidney epithelial cells (6) and recruiting the Src family kinases, Hck and c-Fgr, to the CXCR-1 receptor in neutrophils (7). Recent reports also indicate that β-arrestins can interact directly with component kinases of the extracellular signal-regulated kinase 1/2 (ERK) and c-Jun N-terminal kinase 3 (JNK) mitogen-activated protein (MAP) kinase cascades (6, 8, 9). β-Arrestins have been shown to form complexes with angiotensin II type 1a receptor (AT1aR), c-Raf-1 and ERK (9), with protease-activated receptor 2 (PAR-2), Raf-1 and ERK1/2 (8), and with neurokinin-1 receptor, c-Src and ERK1/2 (6). β-Arrestin2 can also serve as a scaffold for the component kinases of the JNK3 cascade, facilitating JNK3 activation by binding directly to JNK3 and the MAP kinase kinase kinase, Ask1 (9).

Although it is increasingly clear that β-arrestins can bind to some MAP kinases and facilitate their activation by GPCRs, regulated kinases 1 and 2; G protein, heterotrimeric guanine nucleotide-binding protein; GFF, green fluorescent protein; HA, influenza virus hemagglutinin; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP/ERK kinase; PAR-2, protease activated receptor type 2; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; PI, phosphatidylinositol.

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‡ Both authors contributed equally to this work.
** Investigator with the Howard Hughes Medical Institute.
§§ To whom correspondence should be addressed. Tel.: 919-286-0411; Fax: 919-416-5823; E-mail: luttrell@receptor-biol.duke.edu.
1 The abbreviations used are: GPCR, G protein-coupled receptor; AT1aR, angiotensin II type 1a receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; G protein, heterotrimeric guanine nucleotide-binding protein; GFF, green fluorescent protein; HA, influenza virus hemagglutinin; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP/ERK kinase; PAR-2, protease activated receptor type 2; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; PI, phosphatidylinositol.
little is known about the role of β-arrestins in regulating MAP kinase function. Depending on receptor and cell type, GPCRs have been shown to cause ERK1/2 activation by several distinct mechanisms (10, 11). Some of these, such as the protein kinase A-dependent phosphorylation of the small G protein Rap1 (10, 11), the protein kinase C-dependent activation of Raf isoforms (12), and the calcium- and cell adhesion-dependent activation of the focal adhesion kinase Pyk2 (13, 14), are classical second messenger-dependent pathways. In addition, many GPCRs trigger the “transactivation” of receptor tyrosine kinases, such as the epidermal growth factor (15–17) and platelet-derived growth factor receptors (18, 19). In light of such diversity, the question arises as to whether these different pathways of ERK activation are functionally redundant or whether they serve specialized functions within the cell.

To define better the role of β-arrestins in the regulation of the ERK MAP kinases, we have examined the effect of β-arrestin overexpression on ERK1/2 activation and nuclear signal- ing in COS-7 cells expressing AT1aR. We find that while β-arrestin expression enhances AT1aR-stimulated phosphorylation of ERK, the formation of β-arrestin-ERK complexes leads to cytosolic retention of the activated ERK. By spatially con- straining activated ERK1/2, β-arrestins reduce the ability of AT1aRs to stimulate ERK1/2-dependent transcription. These data suggest that β-arrestins may serve to target activated ERK1/2 to non-nuclear substrates.

**EXPERIMENTAL PROCEDURES**

**Materials**—LipoFectAMINE was from Invitrogen. Monoclonal M2 anti-FLAG affinity agarose was from Sigma. Anti-phospho-ERK1/2 antibody was from Cell Signaling, and anti-ERK1/2 antibody was from Upstate Biotechnology Inc. Goat polyclonal anti-lamin B antibody, rabbit polyclonal anti-actin, anti-retinoblastoma protein, and anti-FLAG antibodies were from Santa Cruz Biotechnology. Texas Red®-conjugated anti-rabbit IgG antibody was from Jackson Immunoresearch. Horseradish peroxidase-conjugated donkey anti-rabbit and anti-goat antibodies were from Amersham Biosciences.

**DNA Expression Plasmids**—The pcDNA3.1 expression plasmid encoding hemagglutinin (HA) epitope-tagged AT1aR and green fluores- cent protein (GFP)-tagged β-arrestins were provided by M. G. Caron (Duke University). The pEGFP-N1 expression plasmid encoding GFP-ERK2 (8) was provided by K. A. DeFea and N. Bunnett (University of California, San Francisco). The pCNG-myc-cRaf-1 expression plasmid and the pEGFP-N1 expression plasmid encoding GFP-epitope- tagged β-arrestins were provided by J. G. Caron (Duke University). The pEGFP-N1 expression plasmid encoding GFP-ERK2 (8) was provided by C. Der (University of North Carolina, Chapel Hill). The pFR-Luc, GAL4-Elk-1, and pRL-tk-luc reporter plasmids were from Promega. The pCNG-myc-cRaf-1 expression plasmid encoding cRaf-1 (0.5 μg/plate), with or without pcDNA3-FLAG-β-arrestin1 or -2 (3 μg/plate). The pCNG-ERK1 plasmid was provided by K. A. DeFea and N. Bunnett (University of North Carolina, Chapel Hill). The pCNG-ACT-Raf-1 expression plasmid encoding cRaf-1 was provided by K. A. DeFea and N. Bunnett (University of North Carolina, Chapel Hill). The pCNG-myc-cRaf-1 expression plasmid encoding cRaf-1 (0.5 μg/plate) and the pRL-tk-luc (2 μg/plate), with or without pcDNA3-FLAG-β-arrestin1 or -2 (3 μg/plate). The pCNG-ERK1 plasmid encoding cRaf-1 was provided by K. A. DeFea and N. Bunnett (University of North Carolina, Chapel Hill).

**Cell Culture and Transfection**—COS-7 cells were from the American Type Culture Collection. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml gentamicin. Transient transfection was performed using LipofectAMINE as described previously (20). Transfected cells were incubated overnight in serum-free growth medium supplemented with 0.1% bovine serum albumin (BSA), 10 mM HEPES (pH 7.4), and 100 μg/ml gentamicin prior to stimulation.

**Inositol Phosphate Determination**—COS-7 cells in 100-mm plates were transfected with the expression plasmid encoding HA-AT1aR (2 μg/plate), with or without plasmids encoding β-arrestin1 or -2 (3 μg/plate) as indicated. One day after transfection, cells were split to 12-well plates and incubated for 18–24 h with mho-1(+) humanisotet at 4 μC/ml in serum-free growth medium. After labeling, cells were washed once with phosphate-buffered saline (PBS) and preincubated for 1 h in PBS at 37 °C. The PBS was removed, and cells were incubated in fresh PBS containing 20 μM LiCl for 20 min. Cells were then stimulated for 1 h with 1 μg/ml angiotensin II. The reactions were terminated by the addition of perchloric acid, and total inositol phosphates were isolated by anion exchange chromatography on Dowex AG1-X8 columns, as described (21).

**Immunoprecipitation and Immunoblotting**—Immunoprecipita- tion of FLAG epitope-tagged β-arrestin was performed following transient transfection of COS-7 cells in 100-mm dishes. Cells were transfected with expression plasmids encoding either HA-AT1aR (2 μg/plate) or cRaf-1 (0.1–1.0 μg/plate) and GFP-ERK (1 μg/plate), with or without plasmids encoding FLAG-β-arrestin1 or -2 (3 μg/plate), as indicated. Stimulation was performed as described in the figure legends. After stimulation, monolayers were washed with PBS, solubilized in 1 ml of glycerol lysis buffer (5 mM Heps, 250 mM NaCl, 10% glycerol (v/v), 0.5% Nonidet P-40 (v/v), 2 mM EDTA, 100 μM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Cells were incubated on ice for 5 min to allow lysis prior to centrifugation at 15,000g for 5 min to pellet the nuclei. The supernatants were then separated on a SuperNATR 75 (Triton X-100) gradient. Pellets containing cell nuclei were washed in lysis buffer without Nonidet P-40 and again pelleted at 500 × g. Both cytosolic and nuclear fractions were solubilized in 2× Laemmli sample buffer, and phospho-ERK1/2 was determined by protein immunoblotting. The purity of nuclear and cytosolic fractions was verified by immunoblotting with antibodies to nuclear lamin B, retinoblastoma protein, and actin.
RESULTS

Paradoxical Effects of β-Arrestin Expression on AT1aR-mediated Phosphatidylinositol Hydrolysis, ERK Activation, and ERK-dependent Transcription—In COS-7 cells transiently expressing the HA-AT1aR, angiotensin II stimulation resulted in a 10.8 ± 2.2-fold increase in total inositol phosphates and an 8.9 ± 2.0-fold increase in phosphorylation of coexpressed GFP-tagged ERK1/2. To determine the effect of β-arrestins on AT1aR signaling, we first compared angiotensin II-stimulated phosphatidylinositol (PI) hydrolysis and GFP-ERK2 phosphorylation in the presence and absence of overexpressed FLAG-β-arrestin1 or FLAG-β-arrestin2. Angiotensin II-stimulated PI hydrolysis reflected Gq-dependent activation of phospholipase Cβ isoforms (22). Thus, overexpression of β-arrestins, which accelerate the uncoupling of GPCRs from their cognate G proteins, would be expected to attenuate angiotensin II-stimulated PI hydrolysis. As shown in Fig. 1A, overexpression of either β-arrestin1 or -2 resulted in a significant reduction in angiotensin II-stimulated inositol phosphate production, as expected.

β-Arrestins have also been shown to bind to Raf-1 and ERK2 (8, 23) and to recruit both kinases to the AT1aR in response to angiotensin II stimulation (23). If β-arrestins function as scaffolds to facilitate AT1aR-mediated ERK activation, then overexpression of β-arrestin might be expected to increase AT1aR-mediated ERK phosphorylation. As shown in Fig. 1B, overexpression of FLAG-β-arrestin1 and FLAG-β-arrestin2 increased angiotensin II-stimulated phosphorylation of GFP-ERK2 in whole cell lysates by 1.5- and 2-fold, respectively. These data are consistent with the hypothesis that β-arrestin binding to the AT1aR plays a dual role. By sterically blocking the GPCR-G protein interaction, G protein-dependent signaling is attenuated. Simultaneously, by recruiting ERK to the agonist-occupied receptor, β-arrestin-dependent activation of the ERK cascade is initiated.

Once activated, ERK1/2 phosphorylates numerous plasma membrane, cytosolic, and cytoskeletal substrates (24). Activated ERK1/2 also translocates to the nucleus where it activates transcription by phosphorylating nuclear transcription factors, such as the ETS family protein Elk-1 (24). To determine the effect of β-arrestin overexpression on ERK-dependent transcription, we employed a luciferase reporter assay system under the control of an Elk1-GAL4 fusion protein. COS-7 cells expressing AT1aRs were cotransfected with plasmids encoding the Elk1-GAL4 fusion protein and a firefly luciferase reporter plasmid under the control of GAL4 DNA-binding element. As shown in Fig. 2A, addition of angiotensin II to these cells increased GAL4-dependent expression of the reporter gene by nearly 5-fold over the basal activity. Pretreatment with the MEK inhibitor PD98059 abolished angiotensin II-induced luciferase production. Treatment of cells with PD98059 completely inhibited angiotensin II-induced ERK1/2 activation, indicating that the stimulation of Elk1-driven transcription in response to angiotensin II was mediated entirely through the activation of endogenous ERK1/2. When AT1aR-mediated transcription was assayed in cells overexpressing β-arrestin, we found that the ability of β-arrestins to enhance AT1aR-mediated ERK phosphorylation did not translate into increased AT1aR-mediated Elk1-dependent transcription. As shown in Fig. 2B, overexpression of FLAG-β-arrestin1 and FLAG-β-arrestin2 inhibited angiotensin II-stimulated transcription of the luciferase reporter by 30 and 60%, respectively.

The Formation of β-Arrestin-ERK Complexes Leads to Cytosolic Retention of ERK1/2 by Inducing the Formation of a β-Arrestin-bound Pool of Activated ERK following AT1aR Stimulation—Despite its ability to enhance angiotensin II-stimu-

![Image](http://www.jbc.org/)

**Fig. 1. Effect of β-arrestin overexpression on angiotensin II-stimulated inositol phosphate production and ERK phosphorylation.** A, COS-7 cells were transfected with plasmids for HA-AT1aR, and either FLAG-β-ARR1 (βARR1), FLAG-β-arr2 (βARR2), or empty vector (control) as described. Angiotensin II-stimulated production of [3H]inositol phosphates (IP) was determined as described. Data shown represent the mean ± S.E. of four independent experiments. The result in each experiment was normalized to the level of inositol phosphate accumulated in control cells. *, p < 0.05 versus control. **, p < 0.01 versus control. B, COS-7 cells were transfected with plasmids for HA-AT1aR, GFP-ERK2, and either FLAG-β-arrestin1 (βARR1), FLAG-β-arrestin2 (βARR2), or empty vector (control) as described. GFP-ERK2, which undergoes growth factor-mediated phosphorylation and nuclear translocation like wild type ERK1/2 (8), was used in these experiments to allow ERK2 phosphorylation in the transfected cell pool to be measured in anti-phospho-ERK immunoblots of whole cell lysates. Serum-starved cells were stimulated with angiotensin II (ANG II) (1 μM) for 5 min, and whole cell lysates were resolved by SDS-PAGE. Phosphorylation of GFP-ERK was detected by protein immunoblotting using an anti-phospho-ERK 1/2 antibody. Equal loading of total GFP-ERK protein was confirmed by probing identical immunoblots with an anti-ERK1/2 antibody (not shown). Data shown represent the mean ± S.E. of six independent experiments. The results in each experiment were normalized to the level of GFP-ERK phosphorylation in stimulated control cells. ***, p < 0.01 versus control. The inset panel shows a representative immunoblot of basal (−) and angiotensin II-stimulated (++) GFP-ERK phosphorylation.

lated ERK1/2 phosphorylation, overexpression of β-arrestins inhibited the AT1aR-mediated transcriptional response. This suggests that β-arrestin-dependent ERK1/2 activation leads to the formation of a discrete pool of activated kinase that is unable to induce a transcriptional response. To determine whether AT1aR stimulation in the presence of β-arrestins led to the activation of a β-arrestin-bound ERK pool, we immunoprecipitated FLAG-β-arrestin after angiotensin II stimulation and assayed the immunoprecipitates for the presence of phosphorylated GFP-ERK2. As shown in Fig. 3, GFP-ERK coprecipitated with β-arrestin when FLAG-β-arrestin1 or FLAG-β-arrestin2 was expressed along with AT1aR and GFP-ERK.
β-Arrestin Regulation of ERK Activation and Localization

FIG. 2. Effect of β-arrestin overexpression on angiotensin II-induced Elk-1-dependent transcription. A, COS-7 cells were transfected with plasmids for HA-AT1A/R, pFR-luciferase reporter, GAL4-Elk-1, and TK-Renilla as described. Serum-starved transfected cells were treated with or without PD98059 (PD) (20 μM) for 20 min before stimulation with angiotensin II (ANG II) (1 μM) for 6 h. Luciferase activity in cell lysates was measured as described. Luciferase activity is expressed as the fold increase relative to unstimulated controls and represents the mean ± S.E. from three separate transfections. *, p < 0.05 versus control response. B, COS-7 cells were transfected with plasmids for AT1A/R, pFR-luciferase reporter, GAL4-Elk-1, TK-Renilla, and either FLAG-β-arrestin1 (βARR1), FLAG-β-arrestin2 (βARR2), or empty vector (control) as described. Angiotensin II-induced luciferase activity was measured as described. Luciferase activity is expressed as the fold increase relative to unstimulated controls and represents the mean ± S.E. from three separate transfections. *, p < 0.05 versus control response. The inset panel in A shows a representative immunoblot of basal (−) and angiotensin II-stimulated (+) phosphorylation of endogenous ERK1/2 with or without preincubation of PD98059 (20 μM) for 20 min.

IP. FLAG

CTRL βARR1 βARR2

B. FLAG

AT1A/R

AT1A/R + βARR1

AT1A/R + βARR2

β-Arrestin 1/2

β-Arrestin 1/2

FIG. 3. Effect of angiotensin II stimulation on binding of GFP-ERK2 to β-arrestin and on phosphorylation of GFP-ERK2 bound to β-arrestins. COS-7 cells were transfected with plasmids for HA-AT1A/R, GFP-ERK2, and either FLAG-β-arrestin1 (βARR1), FLAG-β-arrestin2 (βARR2), or empty vector (control, CTRL) as described. Serum-starved cells were stimulated with angiotensin II (ANG II) (1 μM) for 5 min. The amount of total and phosphorylated GFP-ERK present in FLAG-β-arrestin1 or FLAG-β-arrestin2 immunoprecipitates (IP) was determined by immunoblotting. The immunoblots (IB) shown are representative of four independent experiments.

Consistent with the model of β-arrestin-dependent ERK activation, angiotensin II stimulation markedly increased phosphorylation of the β-arrestin-bound GFP-ERK2.

Stimulation of AT1aRs results in rapid recruitment of β-arrestins from the cytoplasm to the agonist-occupied receptor on the plasma membrane, followed by the trafficking of receptor–β-arrestin complexes into endosomal vesicles (25). Because β-arrestins are cytosolic proteins, we hypothesized that activated ERK1/2 bound to β-arrestin might be unable to enter the nucleus and therefore be unable to stimulate Elk-1-dependent transcription. To test this hypothesis, we performed confocal microscopy on COS-7 cells expressing AT1aR along with either GFP or GFP-β-arrestin2, and we determined the effect of β-arrestin expression on the cellular distribution of activated ERK1/2. Phospho-ERK1/2 was visualized after immunofluorescence staining using rabbit polyclonal anti-phospho-ERK1/2 and Texas Red®-conjugated anti-rabbit IgG. As shown in Fig. 4A, in the absence of stimulation, GFP (top row, green) was diffusely distributed throughout the cell, whereas GFP-β-arrestin2 was confined to the cytosol (3rd row, green), and there was minimal staining of phospho-ERK1/2 (top and 3rd row, red). In cells expressing GFP treatment, for 10 min with angiotensin II resulted in an increase phospho-ERK1/2 in both the cytoplasm and nucleus (2nd row, red) without affecting the distribution of GFP (2nd row, green). In contrast, stimulation caused a distinctive redistribution of both phospho-ERK1/2 and GFP-β-arrestin2 in cells expressing GFP-β-arrestin2. After 10 min of agonist treatment, GFP-β-arrestin2 redistributed into characteristic large endosomal vesicles, which we have shown previously contain internalized AT1aR (23) (bottom row, green). In these cells, phospho-ERK1/2 staining partially colocalized with GFP-β-arrestin2, and there was a relative paucity of antiphospho-ERK1/2 immunofluorescence detected in the nucleus (bottom row, red). These findings suggested that overexpression of β-arrestin resulted in the targeting of a significant fraction of the phospho-ERK1/2 to endosomal vesicles and away from the nucleus.

To assess quantitatively the effect of β-arrestin overexpression on the nuclear translocation of ERK1/2 after angiotensin stimulation, we performed cellular fractionation of AT1aR-expressing cells in the presence and absence of overexpressed FLAG-β-arrestin1 or FLAG-β-arrestin2. The purity of nuclear and cytosolic fractions was verified by immunoblotting for nuclear lamin B, retinoblastoma protein, and actin. As shown in Fig. 4B, angiotensin II stimulation resulted in a marked increase in both cytosolic and nuclear phospho-ERK1/2 within 5 min. The increase in phospho-ERK1/2 was accompanied by a modest increase in nuclear ERK1/2, consistent with translocation of ERK1/2 from cytosol to nucleus upon activation. The effect of overexpressing FLAG-β-arrestin1 or FLAG-β-arrestin2 on angiotensin II-stimulated accumulation of nuclear and cytosolic phospho-ERK1/2 is shown in Fig. 4, C and D. In the presence of excess β-arrestin, nuclear phospho-ERK1/2 was reduced by ~60% (Fig. 4C), whereas the amount of phospho-ERK1/2 retained in the cytosolic fraction approximately doubled (Fig. 4D). Thus, while β-arrestin overexpression facilitated AT1aR-mediated activation of either endogenous ERK1/2 or coexpressed GFP-ERK2, the activated ERK was largely retained in the cytosol. This β-arrestin-dependent targeting of activated ERK away from the nucleus correlates with the observed reduction in ERK-dependent transcription in the presence of excess β-arrestin.

The Formation of β-Arrestin-ERK Complexes Leads to Cytosolic Retention of β-Arrestin-bound Phospho-ERK, and Inhibition of Elk-1-driven Luciferase Transcription following cRaf-1 Expression. The overexpression of β-arrestin has complex effects on AT1aR signal transduction. Although receptor-G protein coupling is reduced, leading to diminished Gβγ-mediated PI hydrolysis (Fig. 1A), the efficiency of angiotensin-stimulated ERK activation is enhanced, possibly due to the greater avail-
ability of β-arrestin “scaffolds” (Fig. 1B). Thus, the inhibition of AT1aR-mediated Elk1-driven luciferase transcription could result either from the cytosolic retention of β-arrestin-bound phospho-ERK1/2, as suggested by the confocal microscopy and cell fractionation data (Fig. 4), or from the attenuation of receptor-G protein coupling, which might provide a necessary costimulus for the nuclear translocation of ERK1/2. To discriminate between these two possibilities, we studied the effect of β-arrestin overexpression on ERK localization and transcriptional activity following receptor-independent activation of ERK by overexpressed cRaf-1.

We have shown previously (23) that overexpression of cRaf-1 causes both an increase in the amount of β-arrestin-bound ERK2 and activation of a β-arrestin-bound ERK2 pool. This effect correlates with the binding of cRaf-1 to β-arrestin-ERK complexes, is dependent on endogenous MEK activity, and is independent of GPCR stimulation. As shown in Fig. 5A (upper panel), cRaf-1 expression resulted in a marked increase in

**Fig. 4.** Effect of β-arrestin overexpression on the cellular distribution of activated ERK1/2 following angiotensin II stimulation. A, COS-7 cells were transfected with plasmid DNA encoding HA-AT1aR along with either GFP or GFP-β-arrestin2. Serum-starved cells were treated with vehicle (−) or angiotensin II (Ang II) (+) for 10 min and prepared for immunofluorescence confocal microscopy as described. The distributions of GFP and GFP-β-arrestin2 (green) and Texas Red®-stained phospho-ERK1/2 (red) are shown in the single channel images. Colocalization of GFP-β-arrestin and phospho-ERK1/2 (yellow) is shown in the overlay images. B–D, COS-7 cells were transfected with plasmids for AT1aR and either FLAG-β-arrestin1, FLAG-β-arrestin2, or empty vector (control, CTRL) as described. Serum-starved cells were stimulated with angiotensin II (1 μM) for 5 min. The cells were then fractionated into cytosolic and nuclear fractions as described. B, cytosolic and nuclear fractions from control cells were prepared, and equal amounts of protein from each fraction were subjected to immunoblotting (IB) with antibodies for nuclear lamin B, retinoblastoma protein (RB), actin, phospho-ERK1/2, and total ERK1/2. C, the nuclear fractions from unstimulated and angiotensin II-stimulated control or FLAG-β-arrestin1- (βARR1) or –2 (βARR2)-overexpressing cells were subjected to immunoblotting with antibody to phospho-ERK1/2. Data shown represent the mean ± S.E. of four independent experiments. The result in each experiment was normalized to the level of endogenous phosphorylated ERK1/2 in stimulated control cells. **, p < 0.01 versus control. The inset panel shows a representative immunoblot with basal (−) and angiotensin II-stimulated (+) endogenous ERK phosphorylation in cells transfected as indicated. D, the cytosolic fractions from control or FLAG-β-arrestin1- or –2-overexpressing cells were subjected to immunoblotting with antibody to phospho-ERK1/2. Data shown represent the mean ± S.E. of three independent experiments. The result in each experiment was normalized to the level of endogenous phosphorylated ERK 1/2 in stimulated control cells. *, p < 0.05 versus control (CTRL). The inset panel shows a representative immunoblot with basal (−) and angiotensin II-stimulated (+) endogenous ERK phosphorylation in cells transfected as indicated.
phosphorylation of coexpressed GFP-ERK2 when measured in whole cell lysates. Under these conditions, the presence of FLAG-β-arrestin1 and FLAG-β-arrestin2 did not alter the overall extent of cRaf-1-dependent ERK1/2 phosphorylation. As shown in Fig. 5A (lower panel), cRaf-1 expression caused a 1.5–3-fold increase in the amount of GFP-ERK2 that coprecipitated with β-arrestin and a dramatic increase in the phosphorylation of β-arrestin-bound GFP-ERK2. Because the level of phospho-GFP-ERK2 in the whole cell lysate remained constant, these data would suggest that a larger proportion of the cellular pool of phospho-ERK was β-arrestin-bound in the presence of overexpressed β-arrestin.

We next examined the effect of β-arrestin overexpression on the cellular distribution and transcriptional activity of ERK1/2 that was activated by cRaf-1 expression. Fig. 5B depicts the effect of FLAG-β-arrestin1 or FLAG-β-arrestin2 on the distribution of nuclear and cytosolic phospho-ERK1/2. Similar to the results obtained when ERK1/2 activation was induced by stimulation of the AT1aR, nuclear phospho-ERK1/2 was reduced by ~50% (upper panel), whereas the amount of phospho-ERK1/2 retained in the cytosolic fraction was modestly increased (lower panel) by the presence of excess β-arrestin. Fig. 5C depicts the effect of FLAG-β-arrestin1 and FLAG-β-arrestin2 expression on cRaf-1-stimulated transcription of the Elk1-driven luciferase reporter. Expression of cRaf-1 alone provoked an 8-fold increase in luciferase activity. In the presence of excess β-arrestin, the effect was dramatically reduced. Because these data were obtained in the absence of GPCR activation, and under conditions where β-arrestin expression is leading to the formation of β-arrestin-phospho-ERK complexes, these data suggest that β-arrestin inhibits ERK-dependent transcription by inhibiting the nuclear translocation of activated ERK, rather than by accelerating GPCR desensitization.

**DISCUSSION**

GPCRs are able to employ several distinct mechanisms to activate the ERK1/2 cascade (26). AT1aRs, for example, can activate ERK1/2 not only via β-arrestin-dependent pathways but also through G protein-dependent signals that sometimes involve cross-talk with classical receptor tyrosine kinases (27, 28). In light of such diversity, the question arises as to whether these different pathways of ERK activation are functionally redundant. Our data suggest that they are not. Rather, the consequences of GPCR-mediated ERK activation appear to be determined to a significant degree by the mechanism of ERK activation.

The amount of phosphorylated GFP-ERK2 present in whole cell lysate (upper panel) as well as the total and phosphorylated GFP-ERK2 present in FLAG-β-arrestin1 or FLAG-β-arrestin2 immunoprecipitates (IP) (lower panel) was determined by immunoblotting (IB). B, COS-7 cells were transfected with plasmids encoding cRaf-1 and either FLAG-β-arrestin1 (βARR1), FLAG-β-arrestin2 (βARR2), or empty vector (control, CTRL) as described. Cells were serum-starved overnight and then fractionated into cytosolic and nuclear fractions. The nuclear fractions (upper panel) and cytosolic fractions (lower panel) from control or FLAG-β-arrestin1- or β2-overexpressing cells were subjected to immunoblotting with antibody to phospho-ERK1/2. Data shown represent the mean ± S.E. from three independent experiments. The result in each experiment was normalized to the level of endogenous phosphorylated ERK1/2 in control cells. The inset panels show representative immunoblots with basal (−) and cRaf-1-stimulated (+) endogenous ERK phosphorylation in cells transfected as indicated. C, COS-7 cells were transfected with plasmids for pFR-luciferase reporter, GAL-4-Elk-1, TK-Renilla, and either FLAG-β-arrestin1, FLAG-β-arrestin2, or empty vector (control, CTRL) in the presence and absence of the cRaf-1 expression vector. cRaf-1-induced luciferase activity was measured as described. Luciferase activity is expressed as the fold increase relative to controls and represents the mean ± S. E. from three separate transfections.
β-Arrestin Regulation of ERK Activation and Localization

In a previous study (23), we demonstrated that β-arrestin2 acts as a scaffold for the Raf-dependent activation of ERK1/2. By using confocal microscopy, we found that AT1aRs, β-arrestin2, and phospho-ERK1/2 colocalize in endosomal vesicles after angiotensin stimulation. In this study, we extend these observations by determining the functional effects of β-arrestin expression on ERK1/2 activation and nuclear signaling in response to angiotensin. Although expression of either β-arrestin1 or β-arrestin2 led to an increase in the overall extent of angiotensin-stimulated ERK1/2 phosphorylation, it was associated with a net decrease in both the size of the nuclear pool of phospho-ERK1/2 and in ERK1/2-dependent transcription of an Elk1 reporter. This effect was attributable to the cytosolic retention of ERK1/2 along with AT1aR-β-arrestin complexes in endosomal vesicles.

As depicted schematically in Fig. 6, we propose that AT1aR stimulation results in the activation of two functionally distinct pools of ERK1/2. In COS-7 cells expressing exogenous AT1aRs, the receptor is present in relative excess over the endogenous β-arrestin pool. Under these conditions, AT1aR-mediated ERK1/2 activation proceeds through predominantly β-arrestin-independent mechanisms. These signals lead to an increase in phosphorylated ERK1/2 in the nucleus and stimulate Elk1-dependent transcription. Overexpression of β-arrestin results in a net increase in AT1aR-mediated ERK1/2 activation; however, the ERK1/2 is retained in the cytosol where it is unable to phosphorylate the nuclear pool of Elk-1. Because β-arrestins serve a dual function, in that they uncouple the AT1aR from its G proteins at the same time that they promote β-arrestin-dependent ERK1/2 activation, overexpression of β-arrestin leads to a net reduction in Elk1-dependent transcription. In this model, the transcriptional response reflects a balance between the nuclear translocation of ERK1/2 activated via G protein-dependent pathways and the cytosolic retention of ERK1/2 activated via the β-arrestin-dependent pathway. By increasing β-arrestin levels, the G protein-dependent signal is suppressed; the β-arrestin-dependent pathway is enhanced, and the balance is shifted in favor of the transcriptionally inactive pathway.

Recent work by DeFea et al. (8) supports the hypothesis that different mechanisms of GPCR-stimulated ERK1/2 activation lead to the formation of functionally distinct pools of ERK1/2. In KNRK cells expressing wild type PAR-2 receptors, stimulation of the receptor results in the β-arrestin-dependent activation of a cytosolic pool of ERK1/2 but does not trigger a mitogenic response. Mutant PAR-2 receptors that cannot bind β-arrestin still activate ERK1/2 but do so through a mechanistically distinct, calcium-dependent pathway. This receptor mutant, unlike the wild type receptor, induces nuclear translocation of ERK and does stimulate cell proliferation.

β-Arrestin-ERK complexes appear to be relatively stable entities, because they can be isolated by both gel filtration (6, 8) and immunoprecipitation (6, 8, 23). By using HA-tagged AT1aR, red fluorescent protein-tagged ERK2, and GFP-β-arrestin2 to follow the cellular distribution of the three proteins expressed in HEK-293 cells, we have found previously (23) that AT1aR, β-arrestin, and ERK all localize to endosomal vesicles after angiotensin II stimulation. Thus, the formation of stable complexes between β-arrestin and activated ERK could lead to cytosolic retention of ERK1/2. Our data are consistent with this hypothesis. In the presence of overexpressed β-arrestin, the cytosolic accumulation of endogenous phospho-ERK1/2 is enhanced; the nuclear translocation of phospho-ERK1/2 is impaired, and the ability of AT1aR to induce ERK1/2-dependent, Elk1-driven transcription is inhibited.

In addition to their role in directly phosphorylating nuclear transcription factors, ERK1/2 are known to phosphorylate numerous plasma membrane, cytoplasmic, and cytoskeletal substrates (24). These include several proteins involved in heptahelical receptor signaling, such as β-arrestin1 (29), G protein-coupled receptor kinase-2 (30, 31), and Gα-interacting protein (32). One potential role of β-arrestin-ERK complex formation could be to target specifically ERK1/2 to non-nuclear substrates involved in the regulation of GPCR signaling or intracellular trafficking. Alternatively, β-arrestin-bound ERK1/2 may phosphorylate other cytosolic proteins involved in transcriptional regulation, such as p90RSK (33), which in turn relay signals to the nucleus. In such a model, transcriptional events mediated directly by the nuclear pool of ERK1/2 would be attenuated, whereas alternate pathways of ERK-dependent transcription would persist, resulting in an altered pattern of transcription following activation of the GPCR.

Original models of GPCR signaling envisioned heterotrimeric G proteins as the sole mediators of signals emanating from the receptor, whereas β-arrestins were viewed primarily as terminators of GPCR signaling. Recent data, indicating that β-arrestins themselves participate in meaningful interactions with signaling proteins, including Src kinases (5, 6, 34) and components of the ERK1/2 (6, 8) and JNK3 (9) MAP kinase cascades, suggest that β-arrestins may also function as GPCR-regulated signal transducers. The formation of β-arrestin-ERK complexes could provide a mechanism for controlling the substrate specificity of the kinase, thereby determining the consequences of GPCR-stimulated ERK activation.

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β-Arrestin Scaffolding of the ERK Cascade Enhances Cytosolic ERK Activity but Inhibits ERK-mediated Transcription following Angiotensin AT1a Receptor Stimulation

Akira Tohgo, Kristen L. Pierce, Eric W. Choy, Robert J. Lefkowitz and Louis M. Luttrell

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