Reciprocal Regulation of Angiotensin Receptor-activated Extracellular Signal-regulated Kinases by β-Arrestins 1 and 2*

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β-Arrestin2 not only plays essential roles in seven membrane-spanning receptor desensitization and internalization but also functions as a signal transducer in mitogen-activated protein kinase cascades. Here we show that the angiotensin II type 1A receptor-mediated activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in HEK-293 cells is increased when the cellular level of β-arrestin1 is down-regulated by RNA interference but is decreased or eliminated when the cellular level of β-arrestin2 is diminished. Such reciprocal effects of down-regulated levels of β-arrestins 1 and 2 are primarily due to differences in the ability of the two forms of β-arrestins to directly mediate ERK activation. These results are the first to demonstrate reciprocal activity of β-arrestin isofoms on a signaling pathway and suggest that physiological levels of β-arrestin1 may act as “dominant-negative” inhibitors of β-arrestin2-mediated ERK activation.

Upon agonist binding, seven membrane-spanning (7MS) receptors are phosphorylated by G protein-coupled receptor kinases, which promotes recruitment of cytosolic β-arrestins to the receptor (1). Binding of β-arrestins not only mediates receptor desensitization by physically interfering with the receptor coupling to its cognate G protein (1, 2) but also initiates receptor internalization by interaction with several elements of the clathrin-coated pit endocytic machinery (3–5). Recently, growing evidence has revealed another function of β-arrestins. This is as a signal transducer and adaptor which scaffolds a variety of signaling molecules, for example, Src family kinases (6–8) and components of mitogen-activated protein kinase (MAPK) cascades, leading to their activation (8–14). Such scaffolding and targeting of activated extracellular signal-regulated kinases (ERKS) to a cytoplasmic vesicular compartment has been demonstrated for angiotensin II type 1A (AT1A) (11, 12), neurokinin 1 (8), proteinase-activated (9), and vasopressin (10) receptors (13). Overexpression of β-arrestins enhances cytosolic ERK activation following stimulation of AT1A or V2 receptors (11–13). In addition, studies using the RNA interference (RNAi) technique have demonstrated that β-arrestin2 mediates G protein-independent ERK1/2 activation following AT1A receptor stimulation (15), as well as that β-arrestin2 is required for CXCR4 chemokine receptor-mediated ERK1/2 and p38 activation (16).

The arrestin family consists of two arrestins expressed exclusively in retina (visual and cone arrestins) and the ubiquitously expressed β-arrestins 1 and 2, also known as arrestin 2 and 3, in mammals (17–19). β-Arrestins 1 and 2 are ~70% sequence identical; however, they may have somewhat different properties in terms of their classical functions of desensitization and sequestration of 7MS receptors. For example, β-arrestin2 has 6-fold greater affinity for clathrin than β-arrestin1 in vitro (3). AP-2 also binds preferentially to β-arrestin2 in yeast two-hybrid assays (4). In addition, several agonist-stimulated 7MS receptors appear to more efficiently recruit β-arrestin2 than β-arrestin1 (20). The non-classical G protein-coupled receptor, frizzled4, as well as the type III transforming growth factor β receptor, have recently been shown to recruit β-arrestin2, but not β-arrestin1, resulting in their internalization (21, 22). On the other hand, the proteinase-activated receptor 1 and the metabolic glutamate receptor 1a appear to exclusively utilize β-arrestin1 (23, 24). In the case of MAPK signaling, overexpression of β-arrestin2, but not β-arrestin1, enhances c-Jun amino-terminal kinase 3 (JNK3) phosphorylation stimulated by ASK1 (10). In the case of ERK, depletion of β-arrestin2 levels alone by RNAi is sufficient to abolish G protein-independent ERK1/2 activation (15), suggesting that β-arrestin1, at physiological levels, cannot replace the function of β-arrestin2 to transduce signals to ERK1/2. Data such as these prompted us to carefully examine the roles of β-arrestins 1 and 2, expressed at physiological levels, in signal transduction to ERK from the 7MS AT1A receptor.

EXPERIMENTAL PROCEDURES

Materials—The radiolaabeled compounds, [3H]-[Tyr]angiotensin II (Ang II), and myo-[3H]inositol were obtained from PerkinElmer Life Sciences. Human Ang II was purchased from Peninsula Laboratories, and [Sal]3-Ile3-Ile3-[3H]Ang II (Sal-Ang-Va-Ile-Ile-His-Pro-Ile) was provided by S. S. Karnik (Lerner Research Institute). Epidermal growth factor (EGF), phorbol 12-myristate 13-acetate (PMA), and Ro-31–8425 were purchased from Calbiochem (San Diego, CA). GeneSilencer transfection reagents were from Gene Therapy Systems (San Diego, CA). All other reagents were purchased from Sigma. The pcDNA3.1 expression plasmid encoding the hemagglutinin (HA) epitope-tagged AT1A receptor was provided by M. G. Caron (Duke University).

Synthesis of Small Interfering RNAs (siRNAs)—Chemically synthesized, double-stranded siRNAs, with 19-nucleotide duplex RNA and 2-nucleotide 3′–5′ link overhangs were purchased from Xeragon (Germantown, MD) in deprotected and desalted form. The siRNA sequences targeting β-arrestin 1 and β-arrestin 2 are 5′-AAAGCCUCUUCC

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**Fig. 1. Differential effects of siRNA-inhibited expression of β-arrestins 1 and 2 on ERK activation following AT	extsubscript{1A} receptor stimulation.** A, quantification of β-arrestins 1 and 2 in HEK-293 cells. Endogenous β-arrestins 1 and 2 in cellular extracts (15–90 μg) prepared as described under "Experimental Procedures," and known quantities of purified recombinant β-arrestins 1 and 2 were visualized by immunoblotting with the F4C1 antibody. Concentrations of β-arrestins 1 and 2 were quantitated by densitometry. The β-arrestin levels are plotted as ng of β-arrestin per μg cell protein, and data are expressed as the mean ± S.E. from five independent experiments. B–D, HEK-293 cells were transfected with AT	extsubscript{1A} receptor-encoding plasmids and the indicated siRNAs simultaneously. Three days after transfection, ~60% confluent cells were serum-starved 4–6 h and then stimulated with indicated concentrations of Ang II (C) or EGF (D) for 5 min at 37 °C. After stimulation, cellular extracts were prepared as described under "Experimental Procedures." Equal amounts of protein (10 μg) in each sample were used to visualize expression of β-arrestins (B) and phosphorylation of ERK1/2 (C, D) by immunoblotting. The extent of ERK phosphorylation was determined by densitometry. C, each data point is expressed as percent of the maximal phosphorylation of ERK1/2 in response to 10^{-8} m AngII in control (CTL) siRNA-transfected cells and represents the mean ± S.E. from at least five independent experiments. Dose-response curves were obtained using GraphPad Prism software. A representative immunoblot is shown on the left, in which the lower panel shows equal amounts of ERK2 loaded in each sample. NS, non-stimulated. D, values are shown as percent of EGF-promoted phosphorylation of ERK1/2 in CTL siRNA-transfected cells and represent the mean ± S.E. from three independent experiments. A representative immunoblot is shown on the left.

UGCACCGGAGAU-3′ and 5′-AAGGACCCGAAGUGUGUUGUG-3′, corresponding to the positions 439–459 and 148–168 relative to the start codon respectively (25). A non-silencing RNA duplex (5′-AAUUC- UGCCACGGUUGUUG-3′), as the manufacturer indicated, was used as a control.

**Cell Culture and Transfection—**Human embryonic kidney (HEK)-293 cells were maintained as described previously (20). Forty to fifty percent confluent, slow growing early passage (+15) cells in 100-mm plates were transfected simultaneously with 20 μg of siRNA and 2 μg of the pcDNA3.1-HA-AT	extsubscript{1A} receptor plasmid using the GeneSilencer Transfection reagent as described previously (25). Forty-eight hours after transfection, cells were divided into poly-L-lysine-coated 12-well plates (Becton Dickinson Labware, Bedford, MA) for receptor binding or inositol phosphate determination or 6-well plates to prepare cellular extracts. All assays were performed 3 days after transfection. AT	extsubscript{1A} receptor expression was determined by radioligand binding assays, as described previously (26), and was 200–300 fmol/mg of protein in all experiments.

**Preparation of Cellular Extracts and Immunoblotting—**Cells were solubilized in a lysis buffer containing 5 mM HEPES (pH 7.4), 0.5% Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml peptatin A, 100 μg benzamidine and then clarified by centrifugation at 4 °C for 10 min at 20,000 × g. Equal amounts of proteins were separated on 4–20% (for ERK1/2) or 10% (for β-arrestins 1 and 2) Tris-glycine polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes for immunoblotting. Phosphorylated ERK1/2, total ERK2, total ERK1/2, and β-arrestins were detected by immunoblotting with rabbit polyclonal anti-phospho-p44/42 MAPK (Cell Signaling, 1:2,000), anti-ERK2 (Santa Cruz, 1:2,000) or anti-MAP kinase 1/2 (Upstate Technology Inc., 1:10,000), and anti-β-arrestin (A1CT, 1:3,000) or mouse monoclonal anti-β-arrestin (F4C1, 1:2,000) (27) antibodies, respectively. Chemiluminescent detection was performed using the SuperSignal West Pico reagent (Pierce) and Phosphorylated ERK1/2 and β-arrestin immunoblots were quantified by densitometry with a Fluor-S Multi-Imager (Bio-Rad).

**Recombinant β-Arrestin Purification—**Recombinant rat β-arrestins 1 and 2 were purified from Escherichia coli using a modification of previously published chromatographic procedures (17).

**Inositol Phosphate (IP) Determination—**Cells were incubated overnight at 37 °C in labeling medium (1 μCi of myo-[3H]inositol in 0.5 ml of minimal essential medium with 10% fetal bovine serum per well). Cells were washed with 20 mM HEPES containing 20 mM LiCl for 15–20 min at 37 °C and then treated with agonist for 20 min. Total IPs were extracted and separated as described previously (28). IP production was normalized to total uptake of myo-[3H]inositol in each sample.

**RESULTS AND DISCUSSION**

Using the RNAi technique, we previously demonstrated that β-arrestin2 plays a critical role in G protein-independent ERK1/2 activation by the AT	extsubscript{1A} receptor in HEK-293 cells (15, 25). However, this receptor has been shown to interact equally well with β-arrestins 1 and 2 (20). Moreover, the cellular level of β-arrestin1 (~0.13 ng/μg of cell lysate) is about twice as high.
as that of β-arrestin2 (~0.07 ng/μg of cell lysate) in HEK-293 cells (Fig. 1A). Accordingly, we examined the effect of RNAi-mediated suppression of β-arrestin1 expression on ERK1/2 activation following stimulation of transiently expressed AT1 receptor in HEK-293 cells. Fig. 1B shows that siRNAs targeting β-arrestin1 or β-arrestin2, effectively silence expression of each β-arrestin with clear isoform specificity. Suppression of β-arrestin2 expression expression reduces receptor-mediated ERK1/2 activation by 80–90% of that in control siRNA-transfected cells (Fig. 1C). Unexpectedly, down-regulation of β-arrestin1 expression markedly increases receptor-mediated ERK1/2 activation (Fig. 1C). However, transfection of either β-arrestin siRNA shows no significant effect on EGF-stimulated ERK1/2 activation (Fig. 1D), suggesting that siRNA-inhibited expression of β-arrestins specifically affects 7MS receptor-mediated ERK signaling. Taken together, these results indicate that β-arrestins 1 and 2 have opposite roles in AT1 receptor-mediated ERK signaling, acting as a negative and a positive regulator, respectively.

How does decreasing β-arrestin1 levels potentiate AT1 receptor-mediated ERK1/2 activation, which is opposite to the effect of reduced β-arrestin2 expression? We considered two possibilities based on our previous findings that the AT1 receptor can mediate ERK1/2 activation via two independent mechanisms, β-arrestin2-scaffolding and classical G protein-dependent signaling (15). One possibility is that β-arrestin1, but not β-arrestin2, mediates AT1 receptor desensitization of G protein signaling. In this case, down-regulation of β-arrestin1 levels, by impairing desensitization of the activated receptor, should result in augmentation of G protein-dependent ERK1/2 activation. The other possibility is that β-arrestin1 acts as a physiological dominant-negative at the level of the receptor to block the β-arrestin2-scaffolded ERK activation pathway, since the AT1 receptor binds both β-arrestins equally well (20). In this scenario, the decreased level of β-arrestin1 would allow β-arrestin2 to scaffold components in the ERK pathway more efficiently following receptor activation, resulting in potentiation of ERK1/2 activation.

The AT1 receptor is typically coupled to Gq, which leads to generation of IP and diacylglycerol. To determine whether β-arrestins 1 and 2 play differential roles in AT1 receptor desensitization, we measured IP production upon activation of the receptor in control, β-arrestin1, or β-arrestin2 siRNA-transfected cells (Fig. 2). Suppression of either β-arrestin1 or β-arrestin2 expression does not significantly increase maximum AT1 receptor-mediated IP production compared with that in control siRNA-transfected cells. This indicates that down-regulation of either β-arrestin isoform alone is not sufficient to produce supersensitivity of AT1 receptor coupling to G protein. These data are consistent with previous results obtained with mouse embryonic fibroblast lines derived from β-arrestin knock-out mice, in which depletion of both β-arrestins is required to inhibit desensitization of AT1 receptor-mediated IP production (29). Our results therefore suggest that increased AT1 receptor-mediated ERK1/2 activation in β-arrestin1 siRNA-transfected cells is not due to supersensitive coupling of the receptor to G proteins.

We have previously shown that the AngII-stimulated G protein-dependent pathway to ERK activation in HEK-293 cells is sensitive to the PKC inhibitor Ro-31–8425 (15). To confirm that the increase in AT1 receptor-mediated ERK1/2 activation by down-regulation of β-arrestin1 levels is not primarily due to an augmentation of G protein-dependent signaling, we tested effects of this PKC inhibitor on the activation of ERK1/2 (Fig. 3). Pretreatment with the PKC inhibitor results in moderate inhibition in AT1 receptor-mediated ERK1/2 activation in all
siRNA-transfected cells, indicating that as expected they all have some G protein-dependent activation of ERK1/2 by AngII. Similar to previous results (15), silencing β-arrestin2 expression inhibits ~70% of receptor-activated ERK1/2 signals, and the residual signal is completely abolished by pretreatment with the PKC inhibitor, which eliminates PMA-stimulated signals. These results suggest that in these experiments, 70% of the ERK1/2 activation by AngII is mediated by β-arrestin2 and 30% by G proteins. Consistent with this result, pretreatment with the PKC inhibitor blocks only ~30% of the AT1A receptor-mediated ERK1/2 activation in control siRNA-transfected cells (Fig. 3B). These findings indicate that PKC inhibitor-insensitive ERK1/2 activation is a measure of the β-arrestin2-dependent ERK signaling pathway activated by the AT1A Receptor in HEK-293 cells. In β-arrestin1 siRNA-transfected cells, PKC inhibitor-insensitive ERK1/2 activation following AT1A receptor stimulation is approximately twice that in control siRNA-transfected cells (Fig. 3B), suggesting that the increased level of receptor-mediated ERK1/2 activation observed after down-regulation of β-arrestin1 levels is primarily due to an increase in β-arrestin2-dependent signaling.

The mutant ligand SII-AngII has recently been shown to activate ERK1/2 despite its inability to stimulate receptor coupling to G proteins (30). Moreover, it was demonstrated that activation of ERK1/2 in response to SII-AngII is entirely β-arrestin2-dependent (15). Thus, to further assess the mechanism by which the AT1A receptor-activated ERK activity is potentiated by down-regulation of β-arrestin1, we compared receptor-mediated ERK1/2 activation induced by SII-AngII in control, β-arrestin1, and β-arrestin2 siRNA-transfected cells (Fig. 4). Consistent with previous results (15), silencing the expression of β-arrestin2 completely ablates SII-AngII-stimulated ERK1/2 activation. On the other hand, transfection of β-arrestin1 siRNA augments SII-AngII-stimulated ERK1/2 activation more than 2-fold compared with that in control siRNA-transfected cells. These data strongly support the idea that the increase in AT1A receptor-mediated ERK1/2 activation, which occurs in the presence of decreased levels of β-arrestin1 is due to augmented signaling through the β-arrestin2-dependent ERK pathway.

Our results demonstrate that the two ubiquitously expressed arrestins, β-arrestins 1 and 2, play reciprocal roles in AT1A receptor-mediated ERK signaling. The data suggest that β-arrestin1, at physiological levels in HEK-293 cells, acts as a dominant-negative inhibitor that interferes with β-arrestin2-mediated ERK1/2 activation. However, a previous study in COS-7 cells reported that overexpression of β-arrestin1 as well as β-arrestin2 facilitates AT1A receptor-mediated cytosolic ERK1/2 activation (12). It seems plausible that β-arrestin1 is much weaker than β-arrestin2 in scaffolding the ERK activation cascade. Both β-arrestins are known to have similar affinity for the AT1A receptor (30). Thus, at their physiological levels, β-arrestin1 competes with β-arrestin2 for binding to the activated receptor, causing a decrease in the level of β-arrestin2-bound receptor complexes that facilitate ERK1/2 activation much more efficiently. In contrast, at the much higher expression levels, which are achieved by transfection, β-arrestin1 might reach a threshold concentration required to scaffold the ERK cascade. In fact, not only are much higher levels (>10 times) of exogenous β-arrestin1 than β-arrestin2 required to rescue β-arrestin2 siRNA-inhibited ERK1/2 activation by AngII, but low levels of exogenous β-arrestin1 fail to achieve any rescue (data not shown). Several other findings are also consistent with this formulation. Overexpression of β-arrestin1 is less effective than that of β-arrestin2 in augmenting AT1A receptor-mediated cytosolic ERK1/2 activation (12). Furthermore, the present results (Fig. 4), as well as previous ones (15) showing that G protein-independent ERK1/2 activation following stimulation with SII-AngII can be completely ablated by depletion of β-arrestin2 expression, demonstrate that β-arrestin1 at physiological levels cannot transduce the signal to ERK1/2 activation following AT1A receptor activation.

Recently, accumulating evidence indicates a growing number of physiological roles of β-arrestins not only in 7MS receptor-mediated signaling, but also in other non-classical 7MS receptor functions. Several reports have suggested that β-arrestins 1 and 2 are not equivalent in some cases. However, the data presented here are the first to indicate that the two different forms of β-arrestins actually have reciprocal functions in a 7MS receptor-mediated signaling pathway. In this regard, the ratio between β-arrestins 1 and 2 expression in different cell types may play an important role in fine-tuning different signaling pathways. Thus, regulation of the cellular concentration of an apparently physiologically inert β-arrestin (in this case β-arrestin1) may be an important locus of regulatory control of signaling.

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