Stable Interaction between β-Arrestin 2 and Angiotensin Type 1A Receptor Is Required for β-Arrestin 2-mediated Activation of Extracellular Signal-regulated Kinases 1 and 2*

Huijun Wei‡, Seungkirl Ahn‡, William G. Barnes‡, and Robert J. Lefkowitz‡§

From the ‡Howard Hughes Medical Institute, Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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Binding of β-arrestins to seven-membrane-spanning receptors (7MSRs) not only leads to receptor desensitization and endocytosis but also elicits additional signal-processing processes. We recently proposed that stimulation of the angiotensin type 1A (AT1A) receptor results in independent β-arrestin 2- and G protein-mediated extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation. Here we utilize two AT1A mutant receptors to study these independent pathways, one truncated at residue 324, thus removing all potential carboxyl-terminal phosphorylation sites, and the other bearing four mutations in the serine/threonine-rich clusters in the carboxyl terminus. As assessed by confocal microscopy, the two mutant receptors interacted with β-arrestin 2-green fluorescent protein with much lower affinity than did the wild-type receptor. In addition, the mutant receptors more robustly stimulated G protein-mediated inositol phosphate production. Approximately one-half of the wild-type AT1A receptor-stimulated ERK1/2 activation was via a β-arrestin 2-dependent pathway (suppressed by β-arrestin 2 small interfering RNA), whereas the rest was mediated by a G protein-dependent pathway (suppressed by protein kinase C inhibitor). ERK1/2 activation by the mutant receptors was insensitive to β-arrestin 2 small interfering RNA but was reduced more than 80% by a protein kinase C inhibitor. The biochemical consequences of ERK activation by the G protein and β-arrestin 2-dependent pathways were also distinct. G protein-mediated ERK activation enhanced the transcription of early growth response 1, whereas β-arrestin 2-dependent ERK activation did not. In addition, stimulation of the truncated AT1A mutant receptor caused significantly greater early growth response 1 transcriptions than did the wild-type receptor. These findings demonstrate how the ability of receptors to interact with β-arrestins determines both the mechanism of ERK activation as well as the physiological consequences of this activation.

Stimulation of seven-membrane-spanning receptors (7MSRs)1 leads to G protein coupling as well as receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and the recruitment of arrestins. Phosphorylation of 7MSRs has been shown to occur primarily in the carboxyl-terminal tail (C-tail) and to be critical for stable and high affinity arrestin binding (1–3). Binding of arrestins not only mediates 7MSR desensitization by physically preventing the interaction between G protein and receptor but also initiates receptor internalization. Some receptors, such as the AT1A receptor and V2 vasopressin receptor, bind β-arrestins tightly and internalize with them into endosomal vesicles (“class B”) (4). Others, such as the β2-adrenergic receptor, bind β-arrestins with relatively low affinity, dissociate from them in coated pits, and internalize without β-arrestins (“class A”) (4). These patterns are easily distinguishable by confocal microscopy.

Upon agonist stimulation, the AT1A receptor is phosphorylated on its carboxyl terminus by GRKs and protein kinase C (PKC) (5, 6). The primary PKC phosphorylation sites have been mapped to Ser331, Ser335, and Ser348 (5), which do not appear to be involved in β-arrestin binding (3). In addition, there are also three distinct serine/threonine-rich regions in the COOH terminus. The most carboxy-terminal of these regions (Ser346, Ser347, Ser348) appears to play a minimal role in β-arrestin binding (2, 3), whereas mutations in either the most aminoterminal region (Ser328, Ser329, Ser331, Thr332) or the middle region (Ser335, Thr336, Ser338) change the pattern of β-arrestin 2-GFP recruitment from class B to class A, suggesting that these two regions are important for β-arrestin 2 binding (2). Consistent with this, alanine substitutions for Thr332, Ser335, Thr336, and Ser338 of the AT1A receptor result in a marked decrease in agonist-induced β-arrestin 1 association (3). Furthermore, an AT1A mutant receptor truncated at residue 325, which lacks all of the serine and threonine residues of the carboxyl terminus and shows no agonist-dependent phosphorylation (5), lacks the ability to interact with β-arrestin 1 in an agonist dependent manner as assessed by co-immunoprecipitation (3).

Accumulating evidence strongly suggests that β-arrestins also scaffold signaling pathways such as those leading to mitogen-activated protein kinase activation. In particular, β-arrestins have been shown to form complexes with the AT1A, neurokinin receptor 1, and V2 vasopressin receptors, which scaffold and facilitate the activation of the ERK kinase cascade (RAF, MEK, ERK) while targeting the activated ERK to endocytosis by guest on July 25, 2018http://www.jbc.org/Downloaded from
ERK1/2 Activation by AT1A Receptor Mutants

cytic vesicles in the cytoplasm (7–9). We recently showed that stimulation of a mutant AT1A receptor (DRY/AAY) with angiotensin II (Ang II) or a wild-type receptor with an Ang II analog ([Sar1, Ile8]Ang II) fails to activate classical heterotrimic G protein signaling but does lead to β-arrestin 2 recruitment and ERK1/2 activation (10). In addition, depletion of cellular β-arrestin 2 via siRNA completely abolishes such G protein-independent activation of ERK1/2. Thus the DRY/AAY mutant AT1A receptor and [Sar1, Ile8]Ang II mutant ligand appear to be selective for the β-arrestin 2-dependent G protein-independent pathway leading to ERK1/2 activation. Here we set out to develop corresponding AT1A mutant receptors capable of activating only G protein and independent of β-arrestin 2-mediated signaling; this was accomplished by mutating the carboxy-terminal GRK phosphorylation sites in the receptor that are thought to be necessary for high affinity β-arrestin binding. This approach also permitted us to explore the correlation between β-arrestin/receptor interactions and β-arrestin-mediated ERK1/2 signaling.

EXPERIMENTAL PROCEDURES

Materials—The radiolabeled compounds, [125I]-Tyr-4-Ang II and myo-[3H]inositol, were obtained from PerkinElmer Life Sciences. Human Ang II was purchased from Peninsula Laboratories, Inc. ([Sar1, Ile8]Ang II) Ang II was synthesized in the Cleveland Clinic core synthesis facility (Cleveland, OH). Ro-31-8425 was purchased from Calbiochem. Chemically synthesized double-stranded siRNAs corresponding to human β-arrestin 2 and a non-silencing control were described previously (11). GeneSilencer transfection reagents were from Gene Therapy Systems (San Diego, CA). All other reagents were purchased from Sigma. The pcDNA3.1 expression plasmid encoding hemagglutinin (HA) epitope-tagged AT1A receptors was provided by M. G. Caron (Duke University). β-Arrestin 2-GFP was provided by Sudha Shenoy.

Construction of AT1A Receptor Mutants—Mutant AT1A receptors were generated by mutagenesis PCR using pcDNA3.1-HA-AT1A receptor as a template and a QuickChange multisite-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The primers for TSTS/A and 324/H9004 mutant receptors were 5′-p-CTCAAGCCACGCTGTGCGGAGTAGCCTAGTCGTTGCATCGGTCCTC-3′ and 5′-p-CACCCTGTTGCTGTA (antisense) In each experiment, reverse transcriptase and activate the DNA polymerase. Forty-five transcription-PCR reactions for both EGR-1 and GAPDH were performed in triplicate for each RNA sample. The average threshold cycle values for EGR-1 were normalized to the threshold cycle value of GAPDH and converted to a linear scale.

RESULTS

To generate mutant AT1A receptors that are defective in their ability to associate with β-arrestin 2, we mutated the carboxy-terminal sites in the receptor that must be necessary for high affinity β-arrestin binding. The two mutant AT1A receptors created were TSTS/A, which has alanine substitutions for Thr42, Ser315, Thr336, and Ser338, and 324A, which truncates the AT1A receptor from residue 325 and lacks all of the serine and threonine residues at the carboxyl terminus (Fig. 1A). To test whether TSTS/A and 324A mutant receptors were defective in their ability to associate with β-arrestin 2, we examined the ability of wild-type, TSTS/A, and 324A mutant AT1A receptors to interact with β-arrestin 2-GFP upon agonist stimulation using confocal microscopy. Ang II induced translocation of β-arrestin 2-GFP to endocytic vesicles (class B pattern) (Fig. 1B), demonstrating a stable interaction between β-arrestin 2-GFP and internalized AT1A receptors (2). Conversely, in cells expressing TSTS/A mutant receptor, Ang II induced translocation of β-arrestin 2-GFP only to the plasma membrane (class A pattern) (Fig. 1B). This is consistent with the previous report that the mutated serine/threonine sites are important for the high affinity binding of β-arrestin (2, 5). Ang II also induced translocation of β-arrestin 2-GFP to the plasma membrane in cells expressing the 324A mutant receptor but in a pattern that seemed weaker than the classic class A pattern of β-arrestin 2 recruitment (Fig. 1B) (4).

The carboxyl terminus of AT1A receptors is not thought to be critical for G protein coupling (14). Consistent with this, both TSTS/A and 324A mutant receptors still coupled efficiently to G proteins as indicated by inositol phosphate production turnover assay (Fig. 2). In fact, both mutant receptors mediated somewhat greater constitutive and agonist-stimulated IP production than the wild-type receptor. Both the affinity and maximum activity of the mutant receptors were also increased (Fig. (pH 7.5), 0.5% Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 100 μM benzamidine. Proteins were separated on 10% Tris/glycine polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes for immunoblotting. Phosphorylated ERK1/2, total ERK1/2, and β-arrestin 2 were detected by immunoblotting with a phospho-pp44/42 MAPK antibody (Cell Signaling Technology, Inc., 1:3000), an anti-MAPK1/2 (Upstate Biotechnology, Inc., 1:10,000), and a rabbit polyclonal anti-β-arrestin 2 antibody, 32C7 (1:5000), respectively. Chemiluminescent detection was performed using SuperSignal West Pico reagent (Pierce), and phosphorylated ERK1/2 immunoblots were quantified by densitometry with a Fluor-S Multimager (Bio-Rad).

Quantitative Real Time Reverse Transcription-PCR—Total RNA was purified from cells using an RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. Two micrograms of total RNA was then used to generate cDNA using an Omniscript RT kit (Qiagen). For each PCR reaction, cDNAs corresponding to 300 ng of total RNA were used. Real time PCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions on a MX3000P™ real time PCR system (Stratagene). PCR reactions were carried out by incubating reactions at 95 °C for 10 min to inactivate the reverse transcriptases and activate the DNA polymerase. Forty-five transcription-PCR reactions for both EGR-1 and GAPDH were performed in triplicate for each RNA sample. The average threshold cycle values for EGR-1 were normalized to the threshold cycle value of GAPDH and converted to a linear scale.
2). This presumably reflects impaired β-arrestin-mediated desensitization. Thus, both the TSTS/A and 324Δ receptors are defective in their ability to associate with β-arrestin 2 while retaining the ability to interact with G protein.

We have previously proposed that the AT1A receptor can stimulate ERK1/2 activation by two pathways mediated, respectively, by G protein and β-arrestin 2 (10). Because both TSTS/A and 324Δ can still couple to G protein, activation of G protein-mediated ERK1/2 should not be compromised for either of these two mutant receptors. Consistent with this idea, stimulation of wild-type AT1A receptor, TSTS/A, and 324Δ mutant receptors with Ang II all led to robust ERK1/2 activation (Fig. 3, A and B), which, if anything, was increased with the mutant receptors. To determine the role of β-arrestin 2 in the activation of ERK1/2 by these mutant receptors, we compared the effect of β-arrestin 2 siRNA on the ERK1/2 activation induced by the wild-type AT1A receptor, TSTS/A, or 324Δ mutant receptors. Down-regulation of β-arrestin 2 significantly decreased the ERK1/2 activation induced by wild-type AT1A receptor by ~50–60% (Fig. 3, A and C) as reported previously (10). This suggests that about one-half of the ERK1/2 activation by the wild-type AT1A receptor is mediated by β-arrestin 2. In contrast, no significant inhibition in the ERK1/2 activation was observed when either of the mutant receptors was stimulated with Ang II in β-arrestin 2 siRNA-transfected cells (Fig. 3, A and C). These results indicate that ERK1/2 activation by TSTS/A or 324Δ mutant receptors is largely independent of β-arrestin 2 and mediated primarily by G proteins. In addition, these results demonstrate a good correlation between the ability of β-arrestin 2 to interact with the receptors (as observed by confocal microscopy) and the ability of the receptors to mediate β-arrestin 2-dependent ERK1/2 activation.

We have shown previously that the G protein-mediated ERK1/2 activation by the wild-type AT1A receptor in HEK-293 cells requires activation of PKC, whereas the β-arrestin 2-dependent ERK1/2 activation does not (10). If the ERK1/2 activation induced by TSTS/A or 324Δ mutant receptor is mostly dependent on the G protein-mediated pathway, then ERK1/2 activation induced by these mutant receptors should be more sensitive to PKC inhibition than activation induced by the wild-type AT1A receptor. As predicted, pretreatment with the PKC inhibitor Ro-31-8425 inhibited the wild-type AT1A receptor-stimulated ERK1/2 activation by ~55%, whereas it inhibited TSTS/A or 324Δ mutant receptor-stimulated ERK1/2 activation by >80% (Fig. 4, A and B). A combination of β-arrestin 2 siRNA and Ro-31-8425 almost completely abolished the activation of ERK1/2 induced by the wild-type AT1A receptor as well as TSTS/A and 324Δ mutant receptors (Fig. 4, C and D). Ang II-induced EGR-1 transcription is mediated by ERK1/2 in vascular smooth muscle cells and Chinese hamster ovary cells (15, 16). To determine whether Ang II-induced transcription of EGR-1 is ERK1/2-dependent in HEK-293 cells, we tested the effect of U0126, a specific inhibitor of MEK, just upstream of ERK1/2, on Ang II-induced EGR-1 transcription. Pretreatment with U0126 completely abolished Ang II-induced ERK1/2 activation by the wild-type AT1A receptor (data not shown) and inhibited >97.3 ± 12.3% (n = 3) of Ang II-induced EGR-1 transcription, indicating that Ang II-induced transcription of EGR-1 is dependent on ERK1/2 activation in HEK-293 cells. Because mitogen-induced gene expression requires nu-
significant increase in EGR-1 mRNA levels (Fig. 5 previously shown to activate ERK1/2 only via the
by Ang II. Stimulation of wild-type AT1A receptors with Ang II
inhibitor Ro-31-8425 on the induction of EGR-1 transcription
2-dependent phospho-ERK1/2, we tested the effect of the PKC
mediated by the G protein-dependent but not by
mutant AT1A receptors. Three days after transfection, cells were cultured in serum-free media for 4 h and stimulated with the indicated
concentrations of Ang II for 5 min. The activation of ERK1/2 was determined by immunoblottting with a phospho-ERK1/2-specific antibody. The amounts of total ERK1/2 and β-arrestin 2 were determined by stripping the membrane and immunoblotting for total ERK1/2 and β-arrestin 2. B, the relative levels of maximum phospho-ERK1/2 induced by wild-type (WT), TSTS/A, and 324Δ mutant AT1A receptors were quantified and normalized to that of wild-type AT1A receptors (n = 7). C, the levels of maximum phospho-ERK1/2 in control (CTL) siRNA- and β-arrestin 2 (β-arrestin) siRNA-transfected cells were compared by normalizing to the maximum phospho-ERK1/2 signal induced by each corresponding receptor in control siRNA-transfected cells. **, p < 0.01, compared with the phospho-ERK1/2 induced by Ang II in control siRNA-transfected cells.

FIG. 3. The effect of β-arrestin 2 siRNA on ERK1/2 activation induced by wild-type, TSTS/A, or 324Δ mutant AT1A receptors. A, HEK-293 cells were transfected with control (CTL) siRNA or β-arrestin 2 siRNA and expression vectors encoding wild-type, TSTS/A, or 324Δ mutant AT1A receptors. Three days after transfection, cells were cultured in serum-free media for 4 h and stimulated with the indicated
concentrations of Ang II for 5 min. The activation of ERK1/2 was determined by immunoblottting with a phospho-ERK1/2-specific antibody. The amounts of total ERK1/2 and β-arrestin 2 were determined by stripping the membrane and immunoblotting for total ERK1/2 and β-arrestin 2. B, the relative levels of maximum phospho-ERK1/2 induced by wild-type (WT), TSTS/A, and 324Δ mutant AT1A receptors were quantified and normalized to that of wild-type AT1A receptors (n = 7). C, the levels of maximum phospho-ERK1/2 in control (CTL) siRNA- and β-arrestin 2 (β-arrestin) siRNA-transfected cells were compared by normalizing to the maximum phospho-ERK1/2 signal induced by each corresponding receptor in control siRNA-transfected cells. **, p < 0.01, compared with the phospho-ERK1/2 induced by Ang II in control siRNA-transfected cells.

It has been demonstrated previously that β-arrestins facilitate 7MSR-mediated ERK activation but retain activated phospho-ERK1/2 in the cytosol, which is incapable of inducing ERK-dependent transcription (7, 8, 18, 19). Thus, we would expect that G protein-dependent phospho-ERK1/2 translocates to the nucleus, leading to ERK-dependent transcription, whereas the β-arrestin 2-dependent phospho-ERK1/2 remains in the cytosol and cannot activate ERK-dependent transcription. To determine whether the induction of EGR-1 transcription by Ang II is mediated by the G protein-dependent but not by β-arrestin 2-dependent phospho-ERK1/2, we tested the effect of the PKC inhibitor Ro-31-8425 on the induction of EGR-1 transcription by Ang II. Stimulation of wild-type AT1A receptors with Ang II for 1 h led to an almost 22-fold increase in EGR-1 transcripts (Fig. 5). Pretreatment with the PKC inhibitor all but abolished this induction, suggesting that stimulation of EGR-1 transcription by Ang II is mediated by the G protein-dependent phospho-ERK. To further confirm that the β-arrestin 2-dependent phospho-ERK1/2 is unable to enhance transcription of EGR-1, we tested the ability of [Sar1,Ile4,Ile8]Ang II, which we have previously shown to activate ERK1/2 only via the β-arrestin 2-dependent pathway and not by the G protein-dependent pathway (10), to induce EGR-1 transcription. Stimulation of wild-type AT1A receptors with [Sar1,Ile4,Ile8]Ang II did not lead to a significant increase in EGR-1 mRNA levels (Fig. 5A).

The β-arrestin 2-activated phospho-ERK1/2 pool is confined to the cytoplasm (7, 8, 18, 19). Therefore, we expect that β-arrestin 2 siRNA would not decrease the Ang II-induced EGR-1 transcription even though it leads to a significant decline in cellular phospho-ERK1/2 (Figs. 3 and 4). Consistent with this expectation, β-arrestin 2 siRNA did not significantly change the Ang II-induced EGR-1 transcription (Fig. 5B).

The 324Δ and TSTS/A mutant AT1A receptors are defective in their ability to tightly bind β-arrestins (Fig. 1B) and are more capable of stimulating G protein signaling than the wild-type receptor (Fig. 2). Thus, they might be expected to induce EGR-1 transcription even more strongly than the wild-type AT1A receptor. Consistent with our IP production data, a significant increase in the basal EGR-1 transcripts was observed in cells expressing TSTS/A or 324Δ mutant AT1A receptor (Fig. 5C). In addition, stimulation of the 324Δ mutant AT1A receptor with Ang II for 1 h led to a significant increase in the amount of EGR-1 transcripts compared with the amount of EGR-1 transcripts induced by stimulation of the wild-type AT1A receptor (Fig. 5C), whereas TSTS/A induced an intermediate level of EGR-1 transcripts.

DISCUSSION

We have previously proposed that the AT1A receptor can induce ERK1/2 activation through two independent pathways mediated by G protein and β-arrestin 2, respectively (10). Here we provide support for this idea by showing that the nature of the interaction between β-arrestin 2 and the AT1A receptor is determinative for β-arrestin 2-mediated ERK1/2 activation. Using EGR-1 mRNA as an indicator for nuclear ERK1/2 activation, we further show that G protein-mediated ERK1/2 activation induces EGR-1 transcription, whereas β-arrestin 2-mediated ERK1/2 activation does not. Not only is this consistent with previous reports that β-arrestins sequester phospho-ERK1/2 in the cytosol (7, 18, 19), but it is also indicative of a clear functional divergence in the consequence of ERK1/2 activation by the two pathways. Although several cytosolic proteins such as GRK2, Bcl2, and tau are regulated by ERK1/2

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phosphorylation (20–22), the physiological substrates of the cytosolic phospho-ERK1/2 activated by $\beta$-arrestin 2 remain unknown. Nevertheless, it is clear that the $\beta$-arrestin 2- and G protein-mediated signaling pathways have different physiological consequences.

The 324$\Delta$ mutant receptor lacks all the phosphorylation sites in its C-tail and cannot be phosphorylated in response to Ang II stimulation (5). However, our confocal data clearly indicate that this mutant receptor can recruit $\beta$-arrestin 2 to the plasma membrane in an agonist-dependent manner. These results show that $\beta$-arrestin 2 can bind to $AT_{1A}$ receptors in a phosphorylation-independent manner, albeit with lower affinity.
becomes to be required for high affinity binding of β-arrestins (2, 3), several receptors have been reported to interact with β-arrestins through the third intracellular loop. For example, both the third intracellular loop and the C-tail are important for β-arrestin binding to the neurokinin receptor 1 (23), delta opioid receptor (24, 25), and lutropin/choriogonadotropin receptor (26). In each case, Ser/Thr residues in the third intracellular loop also appear to be critical for β-arrestin binding. However, we found that an AT1A mutant receptor with alanine substitutions for all three Ser/Thr residues in the third intracellular loop can still recruit β-arrestin 2-GFP to the plasma membrane in an agonist-dependent manner (data not shown). Together with previous reports that a C-tail truncation mutant of AT1A receptor at residue 325 cannot be phosphorylated upon Ang II stimulation (3), these results suggest that the phosphorylation-independent association of β-arrestin 2 with the AT1A receptor is driven by conformational changes in the receptor, which occur upon agonist binding. Such a conformational change-dependent recruitment of β-arrestins is not unique. For example, the interaction between β-arrestin 2 and the lutropin/choriogonadotropin receptor depends mostly on receptor activation rather than on receptor phosphorylation (27), and an Asp residue in the third intracellular loop is important for β-arrestin 2 binding to this receptor (28). At first glance, the pattern of agonist-dependent recruitment of β-arrestin 2-GFP to the plasma membrane by the 324A mutant receptor is similar to the pattern of β-arrestin 2-GFP recruitment by class A receptors (4). However, the binding of β-arrestin 2 to 324A mutant receptors and the binding of β-arrestin 2 to a class A receptor might be fundamentally different because β-arrestin 2 recruitment by a class A receptor, such as the β2-adrenergic receptor, is primarily dependent on receptor phosphorylation (29).

The inability of TSTS/A and 324Δ mutant receptors to stably interact with β-arrestins resulted in dramatic inhibition of receptor internalization and increased IP production (3). As a consequence, G protein-mediated ERK1/2 activation was augmented, further reducing the role of the β-arrestin 2-dependent pathway. This may explain why the proportion of β-arrestin 2-dependent ERK1/2 activation induced by stimulation of the TSTS/A or 324Δ mutant receptors is so low, making it hard to detect by the β-arrestin 2 siRNA method. Our data further suggest that the low affinity association of β-arrestin 2 with the AT1A receptor, which is not dependent on receptor phosphorylation, is less able to initiate β-arrestin 2-dependent signaling and that a high affinity state of β-arrestin 2 binding, which requires receptor phosphorylation, is necessary for β-arrestin 2-dependent signaling.

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