Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2

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Stimulation of a mutant angiotensin type 1A receptor (DRY/AAY) with angiotensin II (Ang II) or of a wild-type receptor with an Ang II analog ([sarcosine^1,Ile^4,Ile^8]Ang II) fails to activate classical heterotrimeric G protein signaling but does lead to recruitment of β-arrestin 2-GFP and activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (maximum stimulation ≈50% of wild type). This G protein-independent activation of mitogen-activated protein kinase is abolished by depletion of cellular β-arrestin 2 but is unaffected by the PKC inhibitor Ro-31-8425. In parallel, stimulation of the wild-type angiotensin type 1A receptor with Ang II robustly stimulates ERK1/2 activation with ≈60% of the response blocked by the PKC inhibitor (G protein dependent) and the rest of the response blocked by depletion of cellular β-arrestin 2 by small interfering RNA (β-arrestin dependent). These findings imply the existence of independent G protein- and β-arrestin 2-mediated pathways leading to ERK1/2 activation and the existence of distinct "active" conformations of a seven-membrane-spanning receptor coupled to each.

Signaling by seven-membrane-spanning receptors (7MSRs), such as the angiotensin type 1A (AT_{1A}) receptor, is conventionally ascribed to activation of heterotrimeric G proteins. However, over the past several years an increasing number of examples have been described in which such receptors signal in situations in which G protein activation appears to be minimal or absent (reviewed in ref. 1). In particular, several different angiotensin receptor mutants and angiotensin peptides, which are not able to activate G proteins, have been shown to activate the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinases 1 and 2 (ERK1/2) (2–4). Nonetheless, the mechanisms mediating such G protein-independent signaling have remained unknown.

One candidate for a molecule that might mediate such signaling is β-arrestin. Originally discovered in connection with their ability, together with G protein-coupled receptor kinases, to desensitize activated 7MSRs, β-arrestin 1 and 2 have been found recently to act as adaptors in clathrin-mediated endocytosis and as scaffolds for signaling pathways, such as those leading to activation of MAPKs (5–7). β-Arrestin 2 has been shown to form complexes with the AT_{1A}, NK1 (neurokinin 1), and V2 (vasopressin 2) receptors, which scaffold and facilitate activation of the ERK cascade while targeting the activated ERK to endocytic vesicles in the cytoplasm (8–10). Whether the β-arrestin acts distal to or independent of G proteins in such signaling is not known. Accordingly, we set out in the present studies to determine the relationship of G proteins and β-arrestins in ERK activation by the AT_{1A} receptor.

Materials and Methods

Materials. Tissue culture reagents were purchased from Sigma. The radiolabeled compounds [^{125}I-Tyr^4]angiotensin II (Ang II), guanosine 5′-[γ-[^{35}S]thio]triphosphate ([^{35}S]GTPγS), and *myo*-[^{3}H]inositol were obtained from NEN. Human Ang II was purchased from Peninsula Laboratories. [Sar^1,Ile^4,Ile^8]Ang II (Sar, sarcosine) was synthesized in the Cleveland Clinic core synthesis facility. Phorbol 12-myristate 13-acetate (PMA) and Ro-31-8425 were purchased from Calbiochem. Chemically synthesized, double-stranded small interfering RNAs (siRNAs) corresponding to human β-arrestin 2 (bases 148–168 from start codon; target sequence 5′-AAGGACCGCAAAGUGUUUGUG-3′) and a nonsilencing control (sequence 5′-AAGUGGACCCUGUAGAUGGCG-3′) were purchased from Xeragon (Germantown, MD) in deprotected and desalted form. GeneSilencer transfection reagents were obtained from Gene Therapy Systems (San Diego). All other reagents were purchased from Sigma. The pCDNA3.1 expression plasmid encoding hemagglutinin (HA) epitope-tagged AT_{1A} receptor (pcDNA3.1-HA-AT_{1A}R) was provided by M. G. Caron (Duke University). The pCDNA3.1-DRY/AAY mutant AT_{1A} receptor construct was described (11). GFP-tagged β-arrestin 2 (β-arrestin 2-GFP) was made by cloning rat β-arrestin 2 cDNA in frame in pEGFPN1 (Clontech) between the *Hin*dIII and *Apa*I sites.

Cell Culture and DNA Transfection. Human embryonic kidney (HEK)-293 cells were grown in Eagle's minimum essential medium (MEM) with Earle's salts supplemented with 10% (vol/vol) FBS and a 1:100 dilution of a penicillin/streptomycin mixture (Sigma). Cells were transiently transfected in 10-cm dishes with 2 μg of pcDNA3.1-HA-AT_{1A}R or pcDNA3.1-DRY/AAY by using FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Inositol Phosphate (IP) Determination. Transiently transfected HEK-293 cells in 10-cm dishes were plated onto poly-D-lysine-coated 12-well plates (BD Biosciences Labware). To assay for IP production, cells were incubated overnight at 37°C in labeling medium [1 μCi (1 Ci = 37 GBq) of *myo*-[^{3}H]inositol in 0.5 ml of MEM with 10% FBS per well]. Cells were washed once with PBS for 30 min at 37°C, washed with PBS containing 20 mM LiCl for 20 min at 37°C, and then treated with agonist for 20 min. Total IPs were extracted and separated as described (12).

Radioligand Binding. Radioreceptor assays were performed as described previously by using [^{125}I-Tyr^4]Ang II to determine

Abbreviations: Ang II, angiotensin II; AT_{1A}, angiotensin type 1A; ERK1/2, extracellular signal-regulated kinases 1 and 2; β-arrestin 2-GFP, GFP-tagged β-arrestin 2; HEK, human embryonic kidney; IP, inositol phosphate; MAPK, mitogen-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; 7MSRs, seven-membrane-spanning receptors; Sar, sarcosine; [^{35}S]GTPγS, guanosine 5′-[γ-[^{35}S]thio]triphosphate; siRNA, small interfering RNA.

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cell-surface receptor expression (13). Total protein was determined by the Bradford reaction and was used to standardize AT_{1A} receptor expression levels. The receptor expression levels for wild-type and DRY/AAY (Asp-Arg-Tyr/Ala-Ala-Tyr) mutant AT_{1A} receptor are 1.5–2 pmol of receptors per mg of protein in cells used for IP determination and [^{35}S]GTPγS binding assay and 200–300 fmol of receptors per mg of protein in siRNA-transfected cells.

[^{35}S]GTPγS Binding Assay. Transiently transfected HEK-293 cells in 10-cm dishes were washed twice in PBS, collected in ice-cold homogenization buffer (20 mM Tris·HCl, pH 7.4/100 mM NaCl/1 mM EDTA/10 μM GDP/1 mM PMSF/protease inhibitors) and disrupted with a Dounce homogenizer. Crude membranes were prepared by centrifugation at 30,000 $\times$ *g* for 30 min at 4°C. Membranes were resuspended in assay buffer (50 mM Tris·HCl, pH 7.4/100 mM NaCl/5 mM $MgCl_2$/1 mM EDTA/1 mM DTT/10 μM GDP). Membranes (15 μg of protein per assay tube) were incubated in 100 pM [^{35}S]GTPγS in the presence or absence of agonist for 1 h at 30°C. Binding was terminated by addition of 1 ml of 100 μM GDP/GTP mixture and rapid filtration over GF/B (glass fiber) filters. Filters were washed five times with ice-cold water and dried, and their radioactivity was measured with a liquid scintillation counter (Packard).

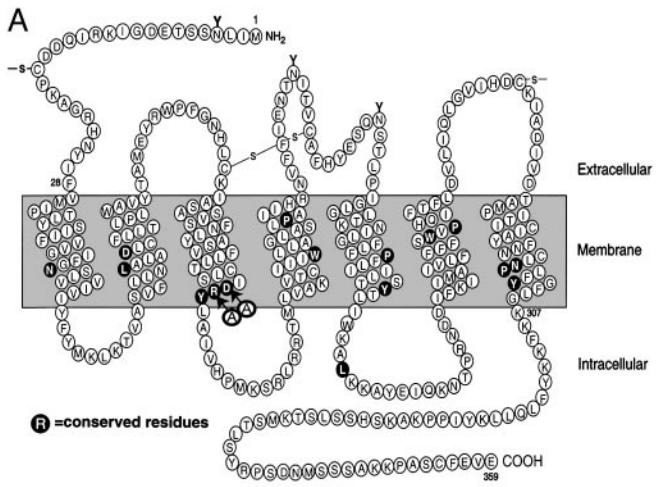
siRNA Transfection. HEK-293 cells were transfected simultaneously with 2 μg of pcDNA3.1-HA-AT_{1A}R or pcDNA3.1-DRY/AAY and 20 μg of β-arrestin 2 siRNA or control siRNA as described (14).

Confocal Microscopy. HEK-293 cells were transiently transfected in 10-cm dishes with 2 μg of pcDNA3.1-HA-AT_{1A}R or pcDNA3.1-DRY/AAY and 0.5 μg of β-arrestin 2-GFP. One day after transfection, cells were split onto collagen-coated 35-mm plastic dishes with glass bottoms (MatTek, Ashland, MA) and cultured overnight at 37°C. Cells were then treated with or without 160 nM Ang II or 30 μM [Sar^1,Ile^4,Ile^8]Ang II for 30 min at 37°C, fixed with PBS containing 5% formaldehyde for 30 min, and washed three times with PBS. Confocal microscopy was performed at $\times$100 magnification with a Zeiss LSM-510 laser-scanning microscope. Images were collected by using 488-nm excitation and a 515- to 540-nm emission filter.

Preparation of Cellular Extracts and Immunoblotting. Cells were solubilized in a lysis buffer [50 mM Hepes, pH 7.5/0.5% Nonidet P-40/250 mM NaCl/2 mM EDTA/10% (vol/vol) 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/100 μM benzamidine] and then clarified by centrifugation for 10 min at 21,000 $\times$ *g*. Equal amounts of supernatant 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 an anti-phospho-p44/42 MAPK antibody (1:3,000; Cell Signaling Technology, Beverly, MA), an anti-MAPK1/2 (1:10,000; Upstate Biotechnology, Lake Placid, NY), and a rabbit polyclonal anti-β-arrestin 2 antibody (A2CT) (1:5,000), respectively. Chemiluminescent detection was performed by using SuperSignal West Pico reagent (Pierce), and phosphorylated ERK1/2 immunoblots were quantified by densitometry with a Fluor-S MultiImager (Bio-Rad).

Results and Discussion

The highly conserved E/DRY (Glu/Asp-Arg-Tyr) motif in the second intracellular loop of 7MSRs is important for G protein coupling. The conserved Arg residue in the rhodopsin E/DRY motif has been shown to interact directly with the G protein to



B

		Kd (nM)
Ang II :	NH2-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-COOH	1.6
[Sar^1, Ile^4, Ile^8]:	Sar-Arg-Val-Ile- Val- His-Pro- Ile-COOH	300

Fig. 1. Secondary structure model of the rat AT_{1A} receptor mutant DRY/AAY (*A*) and sequences of Ang II peptide and its analog [Sar^1,Ile^4,Ile^8]Ang II (*B*). (*A*) Change of DRY to AAY is indicated by arrows. Residues shown in filled circles are conserved in many 7MSRs. DRY/AAY mutant expressed equally well as the wild-type AT_{1A} receptor in all experiments performed. (*B*) The K_d values of Ang II and [Sar^1,Ile^4,Ile^8]Ang II for the wild-type AT_{1A} receptor have been reported by Holloway *et al.* (3). The K_d value for the DRY/AAY mutant has been reported to be 2 nM by Gaborik *et al.* (11). All of these findings have been verified in this study.

catalyze GDP release (15), and its mutation causes impaired signal transduction in many receptors (16, 17). Recently it was reported that the AT_{1A} receptor mutant DRY/AAY (Fig. 1*A*) is severely impaired in $G\alpha_{q/11}$-coupling in COS-7 cells (11). When we transiently expressed this receptor mutant in HEK-293 cells, no detectable inositol 1,4,5-trisphosphate accumulation was observed (Fig. 2*A*), confirming that this mutant receptor lacks functional coupling with $G\alpha_{q/11}$.

Similarly, selected synthetic analogues of Ang II have been described that can support AT_{1A} receptor internalization without activating IP production through $G\alpha_{q/11}$ (Fig. 1*B*) (3). Fig. 2*A* also shows the inability of [Sar^1,Ile^4,Ile^8]Ang II to induce the coupling of the wild-type AT_{1A} receptor to $G\alpha_{q/11}$ in HEK-293 cells by measuring the production of IP. Because the affinity for the AT_{1A} receptor is different between Ang II (K_d = 1.6 nM) and [Sar^1,Ile^4,Ile^8]Ang II (K_d = 310 nM), the production of IP was compared at peptide concentrations causing equivalent levels of receptor occupancy (3). Even at a concentration of 31 μM (100 K_d), [Sar^1,Ile^4,Ile^8]Ang II did not induce detectable accumulation of IP, whereas the equivalent concentration of Ang II maximally activated the production of IP.

The AT_{1A} receptor has been reported to be able to couple to additional G proteins, such as G_i, in some cell lines (18). To determine more generally whether [Sar^1,Ile^4,Ile^8]Ang II can induce the wild-type AT_{1A} receptor to couple to G proteins, and whether Ang II can induce the DRY/AAY mutant receptor to interact with G proteins, we measured agonist-stimulated [^{35}S]GTPγS binding to cell membranes. Whereas Ang II stimulated a robust increase in [^{35}S]GTPγS binding to the AT_{1A} receptor on cell membranes, no increase in [^{35}S]GTPγS binding was detected even in the presence of 562.5 μM [Sar^1,Ile^4,Ile^8]Ang II (Fig. 2*B*). Similarly, Ang II did not stimulate [^{35}S]GTPγS binding to cell membranes prepared from

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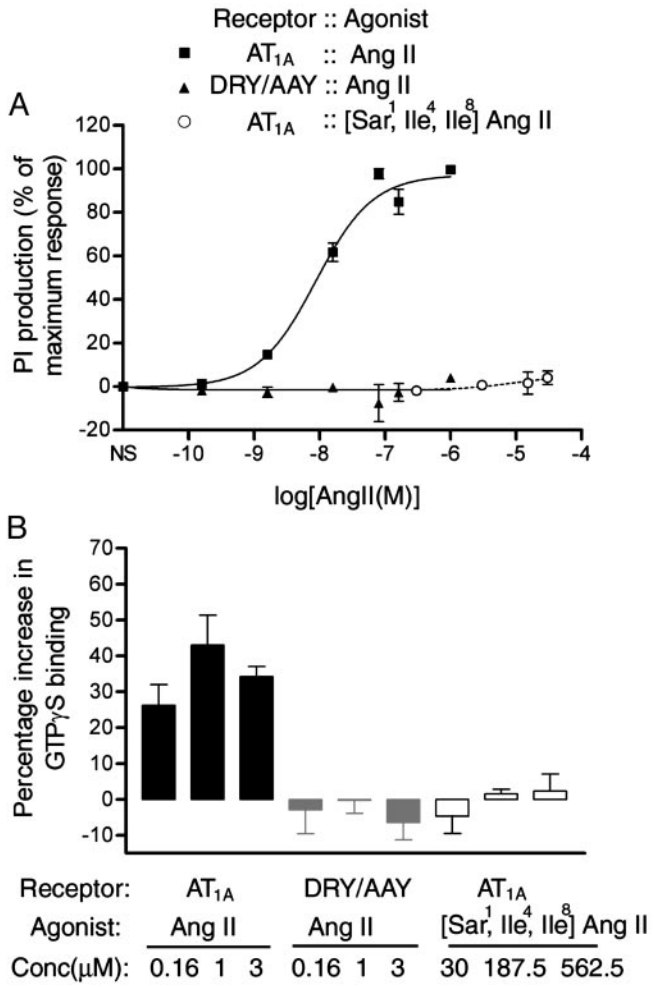

Fig. 2. [Sar^1,Ile^4,Ile^8]Ang II-bound AT_{1A} receptors and Ang II-bound DRY/AAY mutant receptors fail to induce IP accumulation and [^{35}S]GTPγS binding to cell membranes. (*A*) HEK-293 cells were transiently transfected with expression vectors encoding wild-type or DRY/AAY AT_{1A} mutant receptors. Cells were then labeled with *myo*-[^{3}H]inositol for 20 h, and the dose–response of Ang II- and [Sar^1,Ile^4,Ile^8]Ang II-induced accumulation of IP was measured. Each value was then normalized to a percentage of the maximum production of IP mediated by the wild-type receptor on Ang II treatment in each experiment ($n = 4$). (*B*) Cell membranes were prepared, and the agonist-stimulated binding of [^{35}S]GTPγS to cell membranes was measured in the presence of the indicated concentrations [Conc (μM)] of agonists. Data are expressed as percentage increase over the basal values obtained in the absence of agonists ($n = 4$).

DRY/AAY mutant receptor-transfected cells (Fig. 2*B*). Consistent with this finding, mutation in the Arg residue of E/DRY motif in the *N*-formyl peptide receptor has also been shown to abolish G protein coupling (19). Together, these results demonstrate that the Ang II-bound DRY/AAY mutant receptor lacks coupling to G proteins and that [Sar^1,Ile^4,Ile^8]Ang II is not able to induce the wild-type AT_{1A} receptor to couple to G proteins in HEK-293 cells.

To determine whether β-arrestin 2 can interact with Ang II-stimulated DRY/AAY receptors and [Sar^1,Ile^4,Ile^8]Ang II-bound AT_{1A} receptors, we used confocal microscopy to monitor translocation of β-arrestin 2-GFP to receptors on agonist treatment. When β-arrestin 2-GFP was coexpressed with the wild-type AT_{1A} receptor or the DRY/AAY mutant receptor, β-arrestin 2-GFP was distributed uniformly in the cytoplasm of cells (Fig. 3*A*). As shown previously, Ang II induced translocation of

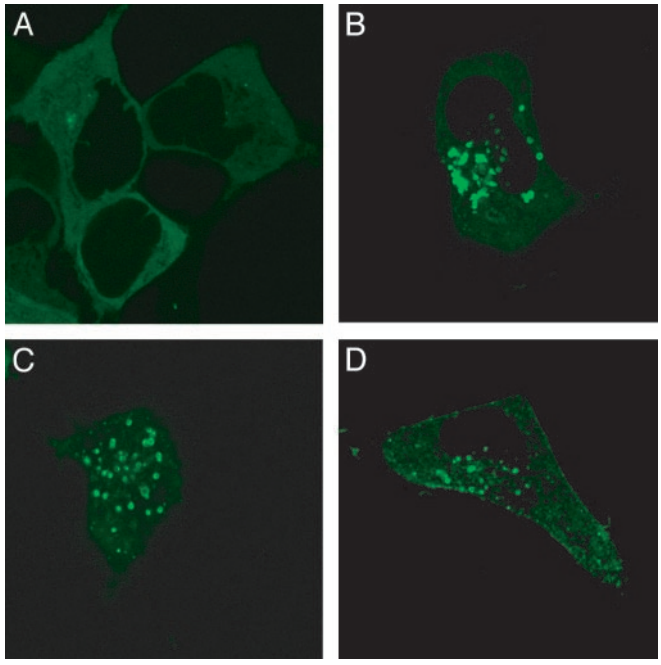

Fig. 3. [Sar^1,Ile^4,Ile^8]Ang II-bound AT_{1A} receptors and Ang II-bound DRY/AAY mutant receptors are able to induce translocation of β-arrestin 2-GFP into endocytic vesicles. HEK-293 cells were transiently transfected with expression vectors encoding β-arrestin 2-GFP and wild-type (*B* and *D*) or DRY/AAY mutant AT_{1A} receptors (*A* and *C*). Cells were not stimulated (*A*), stimulated with 160 nM Ang II (*B* and *C*), or stimulated with 30 μM [Sar^1,Ile^4,Ile^8]Ang II (*D*) for 30 min. Cells were fixed with 5% formaldehyde, and the recruitment of β-arrestin 2-GFP was examined by confocal microscopy. The distribution pattern of β-arrestin 2-GFP in cells that were not stimulated is the same between cells expressing the wild-type and DRY/AAY mutant AT_{1A} receptors. The results shown are representative of three experiments.

β-arrestin 2-GFP to endocytic vesicles in cells expressing AT_{1A} receptors (Fig. 3*B*) because of a stable interaction between β-arrestin 2-GFP and internalized AT_{1A} receptors (20). Ang II-induced translocation of β-arrestin 2-GFP to endocytic vesicles was also detected in DRY/AAY mutant receptor-transfected cells, suggesting that β-arrestin 2 interacts with the DRY/AAY mutant receptor and mediates its internalization on agonist treatment (Fig. 3*C*). Similarly, [Sar^1,Ile^4,Ile^8]Ang II also induced recruitment of β-arrestin 2-GFP to endocytic vesicles in cells expressing wild-type AT_{1A} receptors (Fig. 3*D*), indicating that [Sar^1,Ile^4,Ile^8]Ang II-bound wild-type AT_{1A} receptors can recruit β-arrestin 2 in HEK-293 cells. These results are consistent with previous reports that the DRY/AAY mutant receptor undergoes internalization on Ang II stimulation (11) and that [Sar^1,Ile^4,Ile^8]Ang II can induce AT_{1A} receptor internalization (3).

Stimulation of the wild-type AT_{1A} receptor with [Sar^1,Ile^4,Ile^8]Ang II or the DRY/AAY mutant receptor with Ang II, despite their inability to activate G proteins, led to robust ERK1/2 activation, which was ≈50% of that for the wild-type receptor stimulated by Ang II (Fig. 4*A*). The finding that both the wild-type AT_{1A} receptor stimulated with [Sar^1,Ile^4,Ile^8]Ang II and the DRY/AAY mutant receptor stimulated with Ang II activated ERK1/2 in the absence of detectable heterotrimeric G protein signaling led us to hypothesize that G protein-independent mechanisms might contribute to AT_{1A} receptor-induced ERK1/2 activation. One candidate for mediating G protein-independent 7MSR signals is β-arrestin. β-Arrestins have been shown to bind several signaling proteins, including Src (21), ERK1/2 (8, 9, 22, 23), and JNK3 (24) and to recruit them

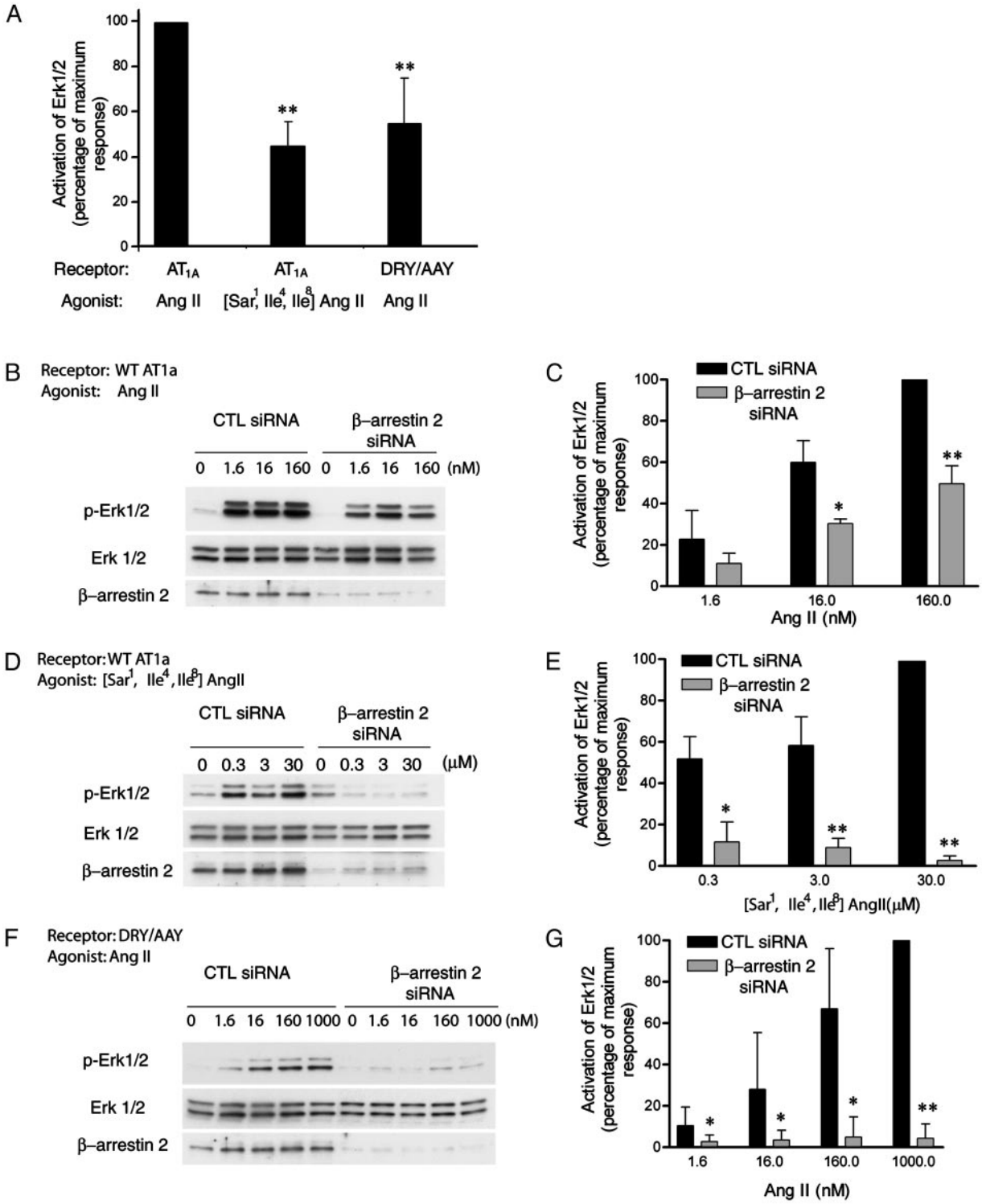

Fig. 4. β-Arrestin 2 mediates ERK1/2 activation induced by [Sar[1],Ile[4],Ile[8]]Ang II-bound AT_{1A} receptors and the Ang II-bound DRY/AAY mutant receptors. (*A*) HEK-293 cells were transfected with control siRNA and expression vectors encoding wild-type or DRY/AAY-mutant AT_{1A} receptors. Three days after transfection, cells were cultured in serum-free medium for 4 h and stimulated with 160 nM Ang II or 30 μM [Sar[1],Ile[4],Ile[8]]Ang II for 5 min. The activation of ERK1/2 was determined by immunoblotting with a phospho-ERK1/2-specific antibody. The levels of phospho-ERK1/2 were then quantified and normalized to the phospho-ERK1/2 signal induced by Ang II-stimulated wild-type AT_{1A} receptors in each experiment. **, $P < 0.01$, compared with the phospho-ERK1/2 induced by Ang II-stimulated wild-type AT_{1A} receptors ($n = 4$). (*B–G*) HEK-293 cells were transfected with control siRNA (CTL siRNA) or β-arrestin 2 siRNA and expression vectors encoding wild-type (WT) (*B–E*) or DRY/AAY-mutant (*F* and *G*) AT_{1A} receptors. The activation of ERK1/2 in cells stimulated with different concentrations of Ang II (*B*, *C*, *F*, and *G*) or [Sar[1],Ile[4],Ile[8]]Ang II (*D* and *E*) was determined as described in *A*. 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. The effect of β-arrestin 2 siRNA on dose-dependent ERK1/2 activation induced by Ang II (*C*, $n = 4$)- or [Sar[1],Ile[4],Ile[8]]Ang II-stimulated wild-type AT_{1A} receptors (*E*, $n = 6$), or the Ang II-stimulated DRY/AAY mutant receptors (*G*, $n = 4$) was plotted by normalizing to the maximum phospho-ERK1/2 signal in each experiment. *, $P < 0.05$; **, $P < 0.01$, compared with the phospho-ERK1/2 signal in the corresponding CTL siRNA-transfected cells.

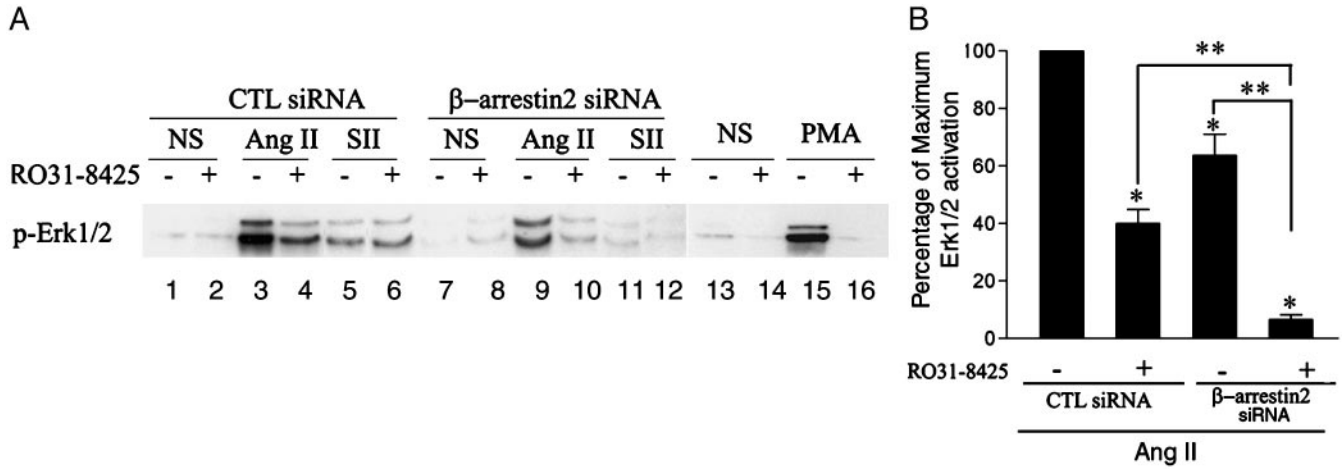

Fig. 5. Effect of the PKC inhibitor Ro-31-8425 on Ang II- and [Sar[1],Ile[4],Ile[8]]Ang II (SII)-induced ERK1/2 activation. (*A*) HEK-293 cells were transfected with control (CTL) siRNA or β-arrestin 2 siRNA, and expression vectors encoding wild-type AT_{1A} receptors. Cells were pretreated with or without Ro-31-8425 for 10 min followed by 5-min stimulation by Ang II or [Sar[1],Ile[4],Ile[8]]Ang II. NS, no stimulation. As a control for the effectiveness of the Ro-31-8425, HEK-293 cells were pretreated with or without Ro-31-8425 for 10 min followed by 5-min stimulation by PMA. The activation of ERK1/2 was determined by immunoblotting with a phospho-ERK1/2-specific antibody (p-Erk1/2). (*B*) The effects of β-arrestin 2 siRNA and Ro-31-8425 on Ang II-induced ERK1/2 activation were compared by normalizing each phospho-ERK1/2 signal to the response induced by Ang II in non-inhibitor-treated cells transfected with control siRNA ($n = 4$). *, $P < 0.01$, compared with the ERK1/2 activation induced by Ang II-stimulated wild-type AT_{1A} receptors; **, $P < 0.01$, compared as indicated.

to 7MSR in an agonist-dependent manner. In the case of the AT_{1A} receptor, β-arrestin 2-bound ERK1/2 has been shown to undergo Ang II-stimulated activation and to localize with the AT_{1A} receptor in endosomal vesicles (8, 9). To assess the importance of β-arrestin 2 in mediating the G protein-independent activation of ERK1/2 documented above, we used the RNA interference technique to down-regulate the expression of endogenous β-arrestin 2 and examined ERK1/2 activation by wild-type Ang II, [Sar[1],Ile[4],Ile[8]]Ang II, and the DRY/AAY mutant receptor. Down-regulation of β-arrestin 2 expression by siRNA transfection decreased the dose-dependent ERK1/2 activation induced by Ang II in cells expressing wild-type AT_{1A} receptors (Fig. 4 *B* and *C*), with the maximum response of ERK1/2 activation decreasing ≈50% compared with that of control siRNA-transfected cells. In contrast, down-regulation of β-arrestin 2 expression by siRNA abolished ERK1/2 activation mediated by the wild-type AT_{1A} receptor on [Sar[1],Ile[4],Ile[8]]Ang II treatment (Fig. 4 *D* and *E*) or by the DRY/AAY mutant receptor stimulated by Ang II (Fig. 4 *F* and *G*). This finding indicates that the G protein-independent activation of ERK1/2 mediated by [Sar[1],Ile[4],Ile[8]]Ang II-bound AT_{1A} receptors or Ang II-bound DRY/AAY mutant receptors is completely dependent on β-arrestin 2. Because our β-arrestin 2 siRNA did not lead to a decrease in β-arrestin 1 expression levels (data not shown), these results also suggest that β-arrestin 2, not β-arrestin 1, is the major form of β-arrestin mediating Ang II-induced G protein-independent ERK1/2 activation.

Because G protein-mediated actions of Ang II are typically transduced by G_q (leading to generation of IP and diacylglycerol and activation of PKC), and because PKC has been shown previously to mediate the Ang II-induced ERK1/2 activation in some physiological target tissues (25), we tested the effects of the PKC inhibitor Ro-31-8425 on ERK1/2 activation (Fig. 5 *A* and *B*). Activation of ERK1/2 by wild-type receptor stimulated with Ang II was inhibited ≈60% by the PKC inhibitor (Fig. 5*A*, lanes 3 and 4, Fig. 5*B*), and the residual activity was virtually eliminated by β-arrestin 2 RNA interference (Fig. 5*A*, lanes 4 and 10, Fig. 5*B*). In contrast, ERK1/2 activation stimulated by [Sar[1],Ile[4],Ile[8]]Ang II was not affected by the PKC inhibitor (Fig. 5*A*, lanes 5 and 6). As expected, the PKC inhibitor blocked Erk1/2 activation by PMA completely (Fig. 5*A*, lanes 15 and 16).

Our results demonstrate the existence of two parallel independent pathways by which ERK can be activated by the AT_{1A} receptor. One pathway is G protein-dependent and involves PKC activation, the other is G protein-independent and is mediated by β-arrestin 2. These findings document that β-arrestin 2 can act as a signal transducer entirely independent of G proteins. However, our results in no way rule out the possibility that under other circumstances G proteins and β-arrestin 2 might act sequentially. One can imagine several scenarios in which such sequential rather than parallel activation of the two pathways might occur. For example, in cases where β-arrestin 2 recruitment is triggered by GRK2/3 (G protein-coupled receptor kinases 2 and 3)-mediated receptor phosphorylation, the role of free $G\beta\gamma$ in this process would require prior G protein activation (26, 27). Alternatively, sequential signaling might result when initial rapid G protein activation of ERK1/2 is subsequently terminated ("desensitized") by β-arrestin 2, which concurrently initiates the β-arrestin 2-dependent phase of ERK1/2 activation.

It seems likely that ERK1/2 activation mediated by β-arrestin 2 will lead to physiological consequences significantly different from those achieved by G protein activation. ERK1/2 activated by G proteins generally accumulates in the nucleus, where it phosphorylates and activates various transcription factors (28). In contrast, ERK1/2 activated by β-arrestin is excluded from the nucleus and is confined to a cytoplasmic compartment (8) where it presumably phosphorylates a distinct set of effectors. Consistent with our findings, ERK1/2 activated by an AT_{1A} mutant receptor uncoupled from G proteins has been shown to localize to the cytoplasm and to be unable to induce activation of Elk1 (2). Similarly, stimulation of the DRY/AAY mutant receptor with Ang II does not induce significant Elk1 activation (11).

An important implication of this work is that the receptor can exist in more than one "active" conformation. Thus, the receptor conformation induced by [Sar[1],Ile[4],Il[8]]Ang II in the wild-type receptor, or by wild-type Ang II in the DRY/AAY mutant receptor, which is competent to activate ERK1/2 via β-arrestin 2, is not able to activate G proteins. Multiple active conformations of 7MSRs have been suggested previously, but have been difficult to document (29, 30). The clear demonstration here of such functionally distinct active conformations, potentially coupled to distinct signaling pathways, gives credibility and impetus

to the development of novel therapeutics that might target only one of several such conformations. Such drugs would likely demonstrate unique biological actions while displaying more limited side effects.

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