Oligomerization of Wild Type and Nonfunctional Mutant Angiotensin II Type I Receptors Inhibits Goαq Protein Signaling but Not ERK Activation*

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The 7-transmembrane or G protein-coupled receptors relay signals from hormones and sensory stimuli to multiple signaling systems at the intracellular face of the plasma membrane including heterotrimeric G proteins, ERK1/2, and arrestins. It is an emerging concept that 7-transmembrane receptors form oligomers; however, it is not well understood which role oligomerization plays in receptor activation of different signaling systems. To begin to address this question, we used the angiotensin II type 1 (AT1) receptor, a key regulator of blood pressure and fluid homeostasis in that specific context has been described to activate ERKs without activating G proteins. By using bioluminescence resonance energy transfer, we demonstrate that AT1 receptors exist as oligomers in transfected COS-7 cells. AT1 oligomerization was both constitutive and receptor-specific as neither agonist, antagonist, nor co-expression with three other receptors affected the bioluminescence resonance energy transfer 2 signal. Furthermore, the oligomerization occurs early in biosynthesis before surface expression, because we could control AT1 receptor export from the endoplasmic reticulum or Golgi by using regulated secretion/aggregation technology (RPD™). Co-expression studies of wild type AT1 and AT1 receptor mutants, defective in either ligand binding or G protein and ERK activation, yielded an interesting result. The mutant receptors specifically exerted a dominant negative effect on Goαq activation, whereas ERK activation was preserved. These data suggest that distinctly active conformations of AT1 oligomers can couple to each of these signaling systems and imply that oligomerization plays an active role in supporting these distinctly active conformations of AT1 receptors.

The 7-transmembrane (7TM)¹ or G protein-coupled receptors such as the angiotensin II type 1 (AT1) receptor constitute the largest group of cell surface membrane receptors and mediate a vast array of biological effects in response to hormones, neurotransmitters, and sensory stimuli. The 7TM receptors activate or interact with numerous signaling proteins including heterotrimeric G proteins, arrestins, adaptor proteins, and extracellular signal-regulated kinases 1 and 2 (ERK1/2) at the intracellular face of the plasma membrane. Although 7TM receptors traditionally have been considered to work as monomeric entities, increasing evidence has shown that these receptors form both homo- and heterodimers or oligomers in vivo and in vitro. Whereas heterodimerization may provide a means to expand pharmacological diversity, the functional role of homodimerization is less well defined (2, 3). 7TM receptor heterodimerization has been shown to modify biological function, receptor trafficking, ligand binding properties, and signal transduction of several 7TM receptors (2, 3). The GABAB “receptor pair” provides an example of two different receptor subunits, the GABAB1 and GABAB2, that both are necessary for receptor surface expression and function (4). The bradykinin B2 has been shown to potentiate AT1 receptor signaling drastically (5), whereas the angiotensin II, type II, receptor inhibits AT1 receptor signaling (6). The β2-adrenergic receptor heterodimerization regulates MAPK signaling efficacy, whereas G protein activation remains unaffected (7).

The role of 7TM receptor homodimerization is experimentally more difficult to assess. Studies of functionally compromised mutants co-expressed with wild type receptors suggest that homodimerization perhaps plays a role in receptor biogenesis, ligand binding, and receptor signaling (2, 3). Mutant receptors of the β2-adrenergic receptor, CCR5, and dopamine D2 receptors have exerted a dominant negative effect on wild type receptor cell surface expression (8–10). In contrast, surface expression of a mutant β2-adrenergic receptor could be rescued by co-expression of WT receptor (11), and internalization of an α-factor receptor that could not internalize was rescued by co-expression of wild type receptor (12). Several studies suggest that dimerization may be important for G protein coupling. Wild type α-factor receptor signaling is inhibited by co-expression of a signaling-deficient receptor (12), and it is possible to inhibit both β2-adrenergic receptor activation and dimerization with a peptide derived from transmembrane domain 6 of the receptor (13). A double cysteine M3 muscarinic receptor that has lost the ability to form covalent dimers and multimers displays an approximately 50-fold reduction in binding affinity compared with wild type and a greater than 10,000-fold reduction in activity, type B; IP, inositol phosphate; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; WT, wild type; Ang II, angiotensin II; CaR, calcium-sensing receptor; Eta, endothelin 1a; HBSS, Hanks’ balanced salt solution.

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¹ The abbreviations used are: 7TM, 7-transmembrane; AT1, angiotensin II type 1; AT1R, AT1 receptor; ERK, extracellular signal-regulated kinase; ER, endoplasmic reticulum; BRET, bioluminescence resonance energy transfer; WT, wild type; GFP, green fluorescent protein; EGFP, enhanced GFP; EGF, epidermal growth factor; GABAB1, γ-aminobutyric acid; type B; IP, inositol phosphate; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; WT, wild type; Ang II, angiotensin II; CaR, calcium-sensing receptor; Eta, endothelin 1a; HBSS, Hanks’ balanced salt solution.
tion in agonist potency (14), and recently mutations that abol-
ished dimerization of the α-factor (15), CCR2, and CCR5 (16)
receptors have been shown to also decrease or abolish agonist-
induced signaling.

Although it is well established that 7T receptors form
homodimers, we are still far from understanding the functional
consequences of this process. Two types of experiments are
currently of great interest: experiments directed at defining the
consequences of this process. Two types of experiments are
examining the role of oligomerization for ligand binding and
signaling. We speculated that the AT1 receptor could be a
means to advance our understanding of how receptor dimer-
ization affects signaling. This receptor has been reported to
engage in both homo- and heterodimerization (5, 6, 17–19),
and it interacts with a diverse array of signaling and scaffold pro-
teins (20). Most important, it has been suggested that AT1
receptor G protein and ERK activation pathways can be sep-
ated. Thus, different angiotensin II peptide analogues and AT1
receptor mutants can activate ERK1/2 without stimulating G
proteins (21–25). In the present study, we examined the effect
of homodimerization on Ang II-induced G protein and
ERK1/2 signaling. We created two receptor mutants defective
in signaling or binding. Most interesting, when co-expressed
with wild type receptors, the mutant receptors inhibit G pro-
tin signaling, whereas ERK activation and β-arrestin recruit-
ment remained unaffected, suggesting that the receptor oli-
gomerization has differential effects on different signaling systems. These results support and expand the notion that AT1
receptor oligomerization plays an important role for activation
of cellular signaling systems.

EXPERIMENTAL PROCEDURES

Materials—Coelenterazine h was purchased from Molecular Probes; DeepBlueCTM, pRLuc, and pGFPv vectors were from BioSignal Packard.
LipofectAMINE(TM) 2000, cell culture media, and serum were from In-
vitrogen. Cell culture plates were from Techno Plastic Products AG (Trasadingen, Switzerland) except for 48-well plates that were obtained from Nunc. Angiotensin II, other ligands, and chemicals were all from Sigma.

cDNA Constructs—The EGFP-tagged bovine β-arrestin 2, as de-
scribed previously (26), was kindly provided by Jeffery Benovic. The
AT1-luc and GFPv and CaR-Luc are described in Ref. 27. ETa-luc was
previously (26), was kindly provided by Jeffery Benovic. The
second portion of the cells was submitted to DeepBlueC

Data Analysis—All pharmacological data were analyzed using
GraphPad Prism. For whole cells radioligand binding data were ana-
lyzed by one-site competitive binding analysis. Luciferase
expression data were analyzed using nonlinear regression curve
fitting. Regarding statistical analysis, all values obtained for variant
receptors were directly compared with those observed for WT receptor. A Bartlett test confirmed that there were no significant differences in

AT1 Receptor Homodimerization Dissects Signaling Pathways

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Bioassay—ETa-luc, MGLu4-luc, and MGLu5-luc were obtained

3.7′-polythyrylization of cellular signaling systems.

(Trasadingen, Switzerland) except for 48-well plates that were obtained from Nunc. Angiotensin II, other ligands, and chemicals were all from Sigma.

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RESULTS

\( \text{AT}_1 \) Receptors Form Constitutive Homodimeric Complexes—We used two different approaches to examine \( \text{AT}_1 \) homodimerization: the BRET\(^2\) technology from Packard Instrument Co., and the regulated secretion/aggregation technology (RPD\(^\text{TM}\)) from ARIAD Pharmaceuticals (30). To analyze homodimerization using BRET\(^2\) experiments, \( \text{AT}_1 \) and control receptors were fused to either Renilla luciferase (Rluc) or green fluorescent protein 2 (GFP\(^2\)) as described previously (27), and these constructs were expressed in COS-7 cells either alone or together (Fig. 1A). When \( \text{AT}_1 \)-luc and \( \text{AT}_1 \)-GFP\(^2\) were co-expressed and Rluc was excited with the substrate DeepBlueC, we detected a robust BRET\(^2\) signal with a value of 0.15 ± 0.01 (Fig. 1A). The BRET\(^2\) signal was not affected by incubation with the receptor agonist Ang II or the inverse agonist Telmisartan (Fig. 1A).

To verify that the BRET\(^2\) signal results from specific protein–protein interactions, we performed two types of experiments. First, we co-expressed three other luciferase-tagged receptors with the \( \text{AT}_1 \)-GFP\(^2\) receptor at the same expression levels (expression data not shown). For this experiment, we chose two 7TM receptors (the endothelin La (Eta) receptor and the calcium-sensing receptor (CaR)) and a truncated version of the EGF receptor dubbed EGF\(_{\text{TR}}\). Although we observed BRET\(^2\) signals in these experiments, these were significantly smaller (0.01–0.08; \( p < 0.05 \)) than that of the \( \text{AT}_1 \) receptors (Fig. 1A). Next, we co-expressed the wild type receptors (\( \text{AT}_1 \), Eta, CaR, and EGF\(_{\text{TR}}\)) together with \( \text{AT}_1 \)-luc and \( \text{AT}_1 \)-GFP\(^2\) receptors at similar expression levels as above to see if they could reduce the BRET\(^2\) signal. Confirming that most of the BRET\(^2\) signal is caused by specific homodimeric protein–protein interactions, the \( \text{AT}_1 \)-WT lowered the \( \text{AT}_1 \)-luc/\( \text{AT}_1 \)-GFP\(^2\) BRET\(^2\) signal significantly to a value of 0.06 ± 0.02; \( p < 0.05 \), whereas none of the three other wild type receptors did (Fig. 1B).

Studies by others have indicated that some 7TM receptors form homodimeric/homo-oligomeric complexes as early as the ER (34–36). To examine whether \( \text{AT}_1 \) receptors dimerize during biosynthesis, we used the regulated secretion/aggregation technology (RPD\(^\text{TM}\)) developed by Rivera et al. (30). In principle, the protein of interest is N-terminally fused to a protein (dubbed \( \text{Fm} \)) that accumulates as aggregates in the ER and Golgi. However, incubation with a synthetic small molecule drug (AP21998) alleviates aggregation and allows the fusion proteins to escape the ER and Golgi in an AP21998-gated fashion. Furthermore, a furin cleavage site has been implemented so that the \( \text{Fm} \) repeats can be enzymatically removed in the Golgi to ensure irreversible disaggregation of the proteins of interest (30). Our idea of using this assay to study homodimerization was to generate a mutant receptor that showed AP21998-gated surface expression but did not activate Ang II-mediated IP accumulation. If this receptor dimerizes with the \( \text{AT}_1 \)-WT, the prediction would be that co-expression of these two receptors would result in AP21998-gated Ang II-mediated signaling. We therefore N-terminally fused a signaling-deficient \( \text{AT}_1 \)R mutated in the DRY motif (D125E/R126E/Y127A/M134A) with four consecutive \( \text{Fm} \) fusion domains, and we expressed this fusion receptor (dubbed \( \text{Fm}_{\text{AT}}-\text{AT}_1 \)-EEA(A)) in COS-7 cells. When exposed to AP21998, the \( \text{Fm}_{\text{AT}}-\text{AT}_1 \)-EEA(A) receptor surface expression approximately doubled (from 8

basal condition in the absence of AP21998 for each receptor combination. For each receptor combination, data were compared with the agonist-induced response in the absence of AP21998 by using a one-tailed paired Student’s \( t \) test (*, \( p < 0.05 \)).

Fig. 1. \( \text{AT}_1 \) receptors form constitutive homo-oligomeric complexes in COS-7 cells. A, the luciferase-tagged receptors were co-expressed with \( \text{AT}_1 \)-GFP\(^2\)-tagged receptors in COS-7 cells, and Ang II or telmisartan was added as denoted in the figure. Equal levels of receptor expression were determined by measurements of luminescence and fluorescence intensities and were used in all experiments (data not shown). The BRET\(^2\) ratios were calculated as described under “Experimental Procedures.” The BRET\(^2\) values observed for each receptor pair were compared with those of the \( \text{AT}_1 \)-luc and \( \text{AT}_1 \)-GFP\(^2\) co-expression by using a one-tailed paired Student’s \( t \) test (*, \( p < 0.05 \)). The data represent the average values (±S.D.) from at least three individual experiments performed in duplicate. B, to further test for specificity, \( \text{AT}_1 \)-luc and \( \text{AT}_1 \)-GFP\(^2\) were co-expressed with various untagged “wild type” receptors in COS-7 cells as indicated. The BRET\(^2\) values observed for each receptor combination were compared with that of the \( \text{AT}_1 \)-luc and \( \text{AT}_1 \)-GFP\(^2\) co-expression using a one-tailed paired Student’s \( t \) test (*, \( p < 0.05 \)). The data represent the average values (±S.D.) from at least three individual experiments performed in duplicate. C, a 2-h incubation with AP21998 (2 μM) up-regulated the \( \text{Fm}_{\text{AT}}-\text{AT}_1 \)-EEA(A) receptor surface expression in COS-7 cells from 8 (±0.02) to 17 (±0.04) fmol per 10^6 cells (\( p < 0.05 \)) as determined by use of a specific binding assay. B_{max} values were compared using a one-tailed paired Student’s \( t \) test and are presented in cpm/well, as a representative of two individual experiments performed in triplicate. D, the effect of a 2-h incubation with AP21998 (2 μM) on the Ang II (10^{-5} M)-induced IP accumulations of the \( \text{Fm}_{\text{AT}}-\text{AT}_1 \)-EEA(A) receptor expressed in COS-7 cells was determined. Data from four individual experiments performed in triplicate represent the normalized average values (±S.D.) with reference to the basal condition in the absence of AP21998. E and F, the \( \text{AT}_1 \)-WT (E) and Eta or CaR wild type receptors (F) were co-expressed with the \( \text{Fm}_{\text{AT}}-\text{AT}_1 \)-EEA(A) receptor in COS-7 cells, and the effects of a 2-h incubation with AP21998 (2 μM) on the agonist (Ang II) = 1 μM; [endothelin II] = 1 μM; [Ca^{2+}] = 50 mM)-induced IP accumulations were determined. Data from four individual experiments performed in triplicate are presented, and they represent the normalized average values (±S.D.) with reference to the variance of the compared groups of data sets. Subsequently, one-tailed, paired Student’s \( t \) tests were performed. All statistically significant results at the \( p < 0.05 \) level are reported.
Co-expression studies of wild type and mutant receptor have so far had three different outcomes. 1) The mutant receptor does not affect wild type signaling; 2) the mutant receptor inhibits wild type signaling either by inhibiting surface expression or the activation of signaling (8–10); or 3) the wild type receptor can functionally rescue the mutant receptor (11, 12, 37). In addition, co-expression of two nonfunctional receptors can create functional receptors (17, 38).

Several studies employing the AT_1 receptor and other 7TM receptors imply the existence of distinct “active” conformations of 7TM receptors. Thus, it has been suggested that some receptor conformations selectively can activate G_protein and ERK activation, whereas a different set of receptor conformations cause G_protein signaling (21–25). We therefore asked the following question: Does receptor dimerization play a role for these states of activation? If so, would a selective binding or signaling-deficient receptor mutant affect all conformations equally? To answer these questions, we generated two mutants as follows: the AT1-EEA(A) that does not activate G_protein but binds Ang II, and AT1-K199A, mutated in the Ang II-binding site. We co-expressed these with AT_1-WT and monitored the effects on signaling, including G_protein-induced IP accumulation, ERK activation, and β-arrestin 2 recruitment.

AT_1 Mutants Exert a Dominant Negative Effect on Wild Type-mediated G Protein Signaling—To be able to detect a dominant negative effect of a mutant or to detect a functional rescue of such a receptor, it is imperative that the assay can detect both decreases and increases in signaling. We therefore transfected increasing amounts of AT_1 receptor cDNA into COS-7 cells, and we measured the Ang II-induced IP accumulation (Fig. 2). We
chose to use 2 μg of DNA per 10-cm culture dish, because the system is very dynamic at this level of receptor expression (Fig. 2A). We next confirmed that the mutants did not activate IP accumulation (Fig. 2B) by testing varying amounts of transfected receptor DNA (0.1–20 μg). No signaling was observed at any receptor amount, although surface expression was confirmed by ligand binding experiments for AT1-EEA(A) and fluorescent antibody staining against an N-terminally incorporated Myc tag for AT1-K199A (data not shown).

To test the effect of the mutants on Go q activation by the wild type AT1 receptor, we co-expressed the receptors. Most interesting, the mutant-WT receptor combination exhibited impaired IP accumulation in response to Ang II as compared with WT receptors alone (p < 0.05) (Fig. 2C), suggesting the mutant receptors exert a dominant negative effect on WT receptor Go q signaling. Co-transfection with Go q did not change this result, suggesting the dominant negative effect was not because of trapping of G protein with p < 0.05 (Fig. 2D). Furthermore, the dominant negative effect was AT1-WT-specific because the IP accumulation response of the ETA and CaR was not affected by the presence of the AT1-receptor mutants (Fig. 2E). Finally, to analyze for a functional rescue, we co-expressed the two mutant receptors defective in either ligand binding or G protein activation; however, none of this combination enhanced IP hydrolysis in response to Ang II (data not shown).

**AT1 Mutants Do Not Affect ERK Activation**—We also tested the mutant receptor effects on Ang II-induced ERK phosphorylation. Most interesting, both of the mutant-WT AT1R combinations induced ERK phosphorylation to the same degree as the wild type AT1 receptor expressed alone (Fig. 3C). In contrast, neither AT1-EEA(A) nor K199A expressed alone activated ERK (Fig. 3B). As mentioned above, the receptors were expressed at levels where we could detect both decreases and increases of ERK-activated signaling (Fig. 3A). We also co-expressed the mutant receptors to look for functional rescue of ERK signaling, but we failed to find any (data not shown). The finding that the mutant-WT combination fully activated ERK signaling also supported the notion that the observed dominant negative effect on Go q signaling was caused by a specific protein-protein interaction at the plasma membrane rather than reduced surface expression of wild type AT1 receptor.

**Compromised AT1 Mutants Do Not Affect Wild Type-induced β-Arrestin 2 Recruitment**—Because the mutant-WT AT1R combinations exhibited dampened G protein activation, but were able to support ERK phosphorylation, we tested the effect of the mutants on AT1-WT-luc Ang II-stimulated β-arrestin 2 recruitment. In general, 7TM receptors are thought to recruit β-arrestin in a G protein-dependent manner, because GRK2 phosphorylation is a preceding event (39). In contrast, some receptors including the AT1 receptor have been shown to support G protein-independent β-arrestin recruitment in specific contexts (21–25). Furthermore, β-arrestin recruitment has been reported to precede and enhance ERK activation (25, 40).

Thus, in line with the ERK activation data, we found the AT1 receptor mutants did not compromise the ability of the AT1-WT-luc to robustly recruit β-arrestin 2 in response to Ang II. To perform this analysis, AT1-luc was co-expressed with EGFP-tagged β-arrestin 2 with and without the receptor mutants (Fig. 4A).

**AT1-R-WT Activation Rescues β-Arrestin 2 Recruitment by AT1-K199A-luc**—We fused Rluc to the C-terminal end of the AT1-K199A receptor to analyze its ability to recruit β-arrestin 2 in the presence of 100 nM Ang II. This receptor did not produce any significant β-arrestin 2 recruitment in response to Ang II (Fig. 4B). However, when we also co-expressed the wild type AT1 receptor, Ang II stimulation produced a robust increase in the BRET signal of 1.7-fold (p < 0.05). In contrast, co-expressing the AT1-WT with other luciferase-tagged receptors (CaR-luc, Eta-luc, and EGF2-luc) did not produce any significant increase in the BRET signal in the presence of Ang II, suggesting that the wild type AT1 receptor specifically rescues β-arrestin 2 recruitment of AT1-K199A (Fig. 4B).
**Experimental Procedures.** Data are depicted as fold responses and represent the average (±S.D.) from three experiments performed in duplicate. 

Further, to analyze whether the AT1-WT receptor would affect the ability of other receptors to interact β-arrestin-2 recruitment by the AT1-K199A-Rluc, we co-expressed AT1-K199A-luc and EGFP-tagged β-arrestin-2 either alone or together with untagged AT1-WT receptor, and we analyzed β-arrestin-2 recruitment as aforementioned. Furthermore, to determine whether the AT1-WT receptor could induce a "functional rescue" of the β-arrestin-2 recruitment by the AT1-K199A-Rluc, we co-expressed AT1-K199A-luc and EGFP-tagged β-arrestin-2 either alone or together with untagged AT1-WT receptor, and we analyzed β-arrestin-2 recruitment as aforementioned. Data are depicted as fold responses and represent the average (±S.D.) from three experiments performed in duplicate. 

**Results,** this system was originally designed to enable regulation of protein secretion in an AP21998-gated fashion. This system should be useful as a tool for others to regulate TM receptor surface expression and to study protein-protein interactions of these receptors. 

**Different States of Activated Receptor Conformations Exist—** By employing co-expression of wild type receptors and mutated receptors defective in either binding or signaling, we found that G protein activation was abolished, whereas ERK activation and β-arrestin-2 recruitment remained intact. Furthermore, we performed careful controls to confirm that both the G protein and the ERK activation assays could detect activity changes if any were present. These observations suggest that the structural demands for ERK activation and β-arrestin recruitment are fulfilled in a complex consisting of wild type and mutant receptors, whereas the conformation required for G protein activation cannot be achieved. Therefore, as supported by previous findings (21–25), distinct active conformations of AT1 receptors that activate different signaling partners exist. Most important, although some of these receptor states may be able to activate all signaling proteins, some states are clearly only capable of activating distinct signaling proteins such as ERKs and β-arrestin.

Two different scenarios can explain the observation that the mutant receptors inhibit wild type receptor-mediated G protein signaling. Either oligomer formation with intact receptors is required for G protein coupling or, alternatively, receptor dimerization may not be necessary for coupling; however, the mutant receptor somehow compromises the ability of the wild type receptors to stabilize the active state required for G protein activation. Presently, we cannot distinguish between these possibilities because we lack a reagent that specifically prevents AT1 receptor dimerization.

Six observations by others suggest that two TM receptors are necessary or are advantageous for G protein coupling. 

1. Applying chemical cross-linking of purified leukotriene B4 receptor BLT1 and Gαi2, β1i, and γ2 proteins in a reconstituted system, Baneres and Pareillo (46) used a combination of mass spectrometry and neutron scattering to establish that only one G protein trimer binds to the dimeric complex of this receptor. 

2. The size of the cytoplasmic surface of a single TM receptor is most likely too small to anchor both the α- and the βγ-subunits. Recently, Palczewski and co-workers (47–50) used their atomic force microscopy data (obtained from native retina membranes in concert with the crystal structures of rhodopsin and the G protein) to propose a model where the monomers in a rhodopsin dimer complex cooperate to dock a single Gαi, and, furthermore, where a neighboring rhodopsin dimer might bridge the Gβγ interaction. 

3. In the heterodimeric GABAAR complex, GABAAR catalyzes G protein activation, whereas the
GABA<sub>B1</sub> subunit binds GABA and improves GABA<sub>B2</sub> G protein coupling but cannot activate the G protein itself, suggesting optimal activation requires both receptors (4, 51). 4) A double cysteine M3 muscarinic receptor that has lost the ability to form covalent dimers and multimers is almost unable to activate the G protein, although the multimer still binds ligand but with reduced affinity (14). 5) Co-transfection of the wild type platelet-activation factor receptor and a mutant receptor (D63N) that does not couple to G protein when expressed alone result in a receptor complex with high constitutive activity. When wild type and mutant receptors were expressed at a ratio of 1/1, the agonist-induced response was greater than that of (D63N) that does not couple to G protein when expressed alone. When the ratio was increased to 1/3, the agonist could no longer induce a response, possibly because the receptors were already desensitized because of the high constitutive activity (37). 6) It has been shown recently that point mutations that abolish dimerization of the α-factor (15), CCR2, and CCR5 (16) receptors also decrease or abolish agonist-induced signaling, although the receptors maintained their high affinity ligand binding.

There are three ways to explain the ERK data. First, dimerization with wild type receptors enables the mutant receptors to activate ERK and recruit β-arrestin. Second, because the mutants did not prevent ERKs and β-arrestin activations, these processes may only need one functional receptor in a complex to activate these molecules, or in fact a monomeric receptor may be capable of this. Third, it seems likely that the mutant receptors affect specific activation states differently, in which case receptor complex might still activate ERK and β-arrestin but not Gα<sub>q</sub>. It is possible that AT<sub>1</sub> receptors activate ERK more robustly than G proteins. Thus, in line with our observations, various AT<sub>1</sub> receptor ligands that activate ERKs but not G protein have been identified, whereas no ligand solely activating G protein has been described (24, 25). In addition, mutant AT<sub>1</sub> receptors that induce β-arrestin recruitment and ERK phosphorylation or internalization, but not G protein activation, have been described (21, 23).

Along with our observation that AT<sub>1</sub>-WT receptor can rescue β-arrestin 2 recruitment by AT<sub>1</sub>-K199A (Fig. 4B), several other studies suggest that a dimeric receptor complex might be needed for interaction with β-arrestin and ERKs. Accordingly, β<sub>2</sub>-adrenergic receptors heterodimerize with AT<sub>1</sub> receptors, and receptor antagonists against either of these receptors abolish both G protein and ERK activation from both receptors (19). Moreover, endocytosis of receptors that do not internalize alone has been functionally rescued by either WT or different receptors in homo- or heterodimeric complexes (12, 52) as well as a mutant M3 receptor that has been reported to exert dominant negative effects specifically on WT M3 receptor ERK activation (53). Finally, the crystal structure of β-arrestin may contain two binding sites for 7TM receptors (54, 55).

Conclusions—We have shown that AT<sub>1</sub> receptors specifically and ligand independently dimerize during biosynthesis before surface expression. Furthermore, AT<sub>1</sub> receptor mutants exerted a dominant negative effect on Gα<sub>q</sub> activation, whereas ERK activation was preserved, suggesting that separate activation conformations of AT<sub>1</sub> receptors can be coupled to each of these signaling systems. These data imply oligomerization plays an active role in supporting distinct active conformations of AT<sub>1</sub> receptors.

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