Apparent Loss-of-Function Mutant GPCRs Revealed as Constitutively Desensitized Receptors†

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ABSTRACT: The DRY motif is a triplet amino acid sequence (aspartic acid, arginine, and tyrosine) that is highly conserved in G protein-coupled receptors (GPCRs). Recently, we have shown that a molecular determinant for nephrogenic diabetes insipidus, the vasopressin receptor with a substitution at the DRY motif arginine (V2R R137H), is a constitutively desensitized receptor that is unable to couple to G proteins due to its constitutive association with β-arrestin [Barak, L. S. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 93−98]. Additionally, the mutant receptors are localized in endocytic vesicles, identical to wild-type receptors stimulated with agonist. In this study, we asked whether the constitutively desensitized phenotype observed in the V2R R137H represents a general paradigm that may be extended to other GPCRs. We show that arginine substitutions in the DRY motifs of the α_{1B} adrenergic receptor (α_{1B}-AR) and angiotensin II type 1A receptor ($AT_{1A}R$) result in receptors that are uncoupled from G proteins, associated with β-arrestins, and found localized in endocytic vesicles rather than at the plasma membrane in the absence of agonists. The localization of the α_{1B}-ARs and AT_{1A}Rs with arginine substitutions can be restored to the plasma membrane by either using selective antagonists or preventing the endocytosis of the β-arrestin−receptor complexes. These results indicate that the arginine residue of the DRY motif is essential for preserving the localization of the inactive receptor complex. Furthermore, constitutive desensitization may underlie some loss-of-function receptor phenotypes and represent an unappreciated mechanism of hormonal resistance.

G protein-coupled receptors (GPCRs)[1] are seven-transmembrane proteins that convert a variety of extracellular signals into physiological responses. The GPCR superfamily is comprised of three subfamilies, the largest being the class I rhodopsin-like receptors. Many common structural and sequence motifs are evident within the receptors of this subfamily (*1*, *2*). One highly conserved signature sequence of amino acids, the DRY motif (aspartic acid, arginine, and tyrosine), is located near the cytoplasmic boundary of the third transmembrane domain and second intracellular loop. Although variations of the motif's first and third residues are often found, the placement of the central arginine residue is conserved with very few exceptions. Both naturally occurring and engineered mutations of the arginine residue frequently impair receptor signaling by decreasing the ability of the receptor to couple to G proteins. Receptors such as the α_{1B}-adrenergic receptor (α_{1B}-AR), the cannabinoid receptor, the CX_3CR1 chemokine receptor, and the vasopressin type II receptor (V2R) that have mutations in the DRY motif arginine residue have been characterized as displaying a loss-of-function phenotype (*3*−*6*). For example, the V2R R137H is decoupled from G proteins and exhibits a markedly reduced ability to activate cyclic AMP. Moreover, the V2R R137H is constitutively phosphorylated in the absence of agonist and is localized in endocytic vesicles rather than at the plasma membrane. The physiological consequence of this mutation is the water losing syndrome nephrogenic diabetes insipidus (*6*, *7*), and its apparent molecular basis is an enhanced affinity of the receptor for β-arrestin proteins (*8*).

Arrestins are cytosolic proteins that preferentially bind to GPCRs that have been phosphorylated by G protein-coupled receptor kinases (GRKs). This interaction competitively inhibits receptor−G protein coupling (*2*). The β-arrestins also function as docking proteins that target the receptor−β-arrestin complex to clathrin-coated pits for endocytosis (*9*, *10*). Since arrestins apparently possess the ability to desensitize nearly all ligand-activated GPCRs, it is probable that the arrestin-mediated constitutively desensitized phenotype observed in the V2R R137H occurs in other GPCRs with analogous substitutions. To test this hypothesis, we chose to evaluate substitutions of the arginine residue in the DRY motif of the α_{1B}-AR and the $AT_{1A}R$.

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[1] Abbreviations: α_{1B}-AR, golden hamster α_{1B}-adrenergic receptor; AngII, angiotensin II; $AT_{1A}R$, rat angiotensin II type 1A receptor; DAG, diacylglycerol; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; IP, inositol phosphate; NE, norepinephrine; PIP_2, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase C; V2R, human vasopressin receptor.

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α_{1B}-ARs and AT_{1A}Rs are known to redistribute themselves from their localization at the plasma membrane to endocytic vesicles upon the stimulation of agonists (*11*, *12*). Additionally, both receptors are known to mediate agonist stimulation by coupling to G_q proteins and activating phospholipase C (PLC). The subsequent formation of secondary messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) induces intracellular calcium release and the activation of protein kinase C (PKC) (*13*, *14*). Whereas previous studies of the AT_{1A}R indicated that substitution of the entire DRY motif led to a loss of receptor signaling (*15*), studies of the α_{1B}-AR have indicated that substitution of the central arginine residue alone (R143) is sufficient (*3*). α_{1B}-ARs with R143 mutations have been previously described as having an up to 300-fold increased affinity for agonist, increased levels of basal phosphorylation, and impaired signaling ability as measured by IP_3 production (*3*).

In this study, we investigated how arginine substitutions in the DRY motifs of the AT_{1A}R and α_{1B}-AR affected their signaling, localization, and desensitization. These two receptors represent each class of rhodopsin-like GPCRs, those that do not associate with β-arrestin in intracellular endosomes upon agonist stimulation (class A, α_{1B}-AR) and those that do (class B, AT_{1A}R) (*16*). Additionally, the DRY motif of both receptors has been demonstrated to be a critical component for maintaining G protein coupling ability (*3*, *15*).

MATERIALS AND METHODS

Materials. Norepinephrine (NE) was obtained from Research Biochemical International (Natick, MA). [^{35}S]GTPγS, [^{3}H]prazosin, and [^{3}H]angiotensin II were from NEN (Boston, MA). Protease inhibitor cocktail tablets were from Roche Molecular Biochemicals (Indianapolis, IN). GDP, phentolamine, and angiotensin II (AngII) were from Sigma. L158,-809 was a generous gift from E. Escher (Université de Sherbrooke, Sherbrooke, PQ). The golden hamster wild-type α_{1B}-AR, R143A, and R143H were a kind gift from S. Cotecchia (Université de Lausanne, Lausanne, Switzerland). GF/B filters and the cell harvester were obtained from Brandel (Gaithersburg, MD). HEK-293 and COS cells were from the American Type Culture Collection (Manassas, VA), and cell culture reagents were from Life Technologies (Rockville, MD) and Cellco (Kensington, MD).

Plasmids and Constructs. The N-terminal HA epitope-tagged constructs were generated by PCR using 5′ primers containing the HA sequence (TACCCATACGACGTCCCAGACTACGCT) followed by the gene sequence and cloned into pcDNA3.1 (Invitrogen) and pEGFP-N3 (CLONTECH) at the *Nhe*I−*Hin*dIII and *Nhe*I−*Sal*I sites, respectively. The α_{1B}-AR R143E, R143H, R143K, and R143N were generated by PCR and inserted into the *Nhe*I−*Sac*II and the *Xho*I−*Pst*I sites of the wild-type α_{1B}-AR in pcDNA3.1/zeo and pEGFP-N3, respectively. The rat AT_{1A}R R126H was generated by PCR and cloned into pcDNA3.1/zeo and pEGFP-N3 at the *Nhe*I−*Hin*dIII and *Nhe*I−*Sal*I sites, respectively. K44A dynamin, Gα_q, GRK, and β-arrestin-GFP were constructed as previously described (*17*, *18*).

Cell Culture and Transfection. HEK-293 cells were grown in Eagle's minimum essential medium with Earle's salt (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and a 1:100 dilution of a penicillin/streptomycin mixture (Sigma). Cells were transiently transfected in 10 cm dishes (Falcon) with 1 μg of receptor, K44A dynamin, Gα_q, or GRK plasmid cDNA in pCDNA3.1 using a modified calcium phosphate coprecipitation method (*19*) for inositol phosphate and binding assays. For confocal microscopy, cells were transiently transfected on collagen (Sigma)-coated 35 mm glass bottom dishes (MatTek, Ashland, MA) with Lipofectamine 2000 and Opti-MEM media (Life Technologies) using a standard method (*20*). Cells for confocal microscopy were transfected with 30 ng of β-arrestin or receptor plasmid cDNA in pEGFP and 250 ng of K44A dynamin, GRK, or receptor in pCDNA3.1.

Receptor Binding. Transiently transfected HEK-293 cells in 10 cm dishes were washed twice in cold binding buffer [MEM with 2% bovine serum albumin (BSA)], incubated for 1 h at room temperature in binding buffer with varying concentrations of [^{3}H]prazosin (from 0.25 to 8 nM), and washed three times in cold binding buffer to remove unbound ligand (a fixed concentration of 8 nM [^{3}H]prazosin was used to measure the level of binding in cells coexpressing K44A dynamin or cultured in phentolamine). The amount of cell-bound [^{3}H]prazosin was measured using a scintillation counter. The level of nonspecific binding was determined in the presence of a 1000-fold molar excess of phentolamine (10 μM). Receptor binding of the AT_{1A}R was carried out as previously described (*21*) with [^{3}H]angiotensin II (0.375−12 nM).

Inositol Phosphate Determination. Transiently transfected HEK-293 cells in 10 cm dishes were plated onto 12-well plates (Falcon) coated with 25 μg/mL poly-D-lysine (Sigma). To assay for inositol phosphate production, cells were incubated overnight at 37 °C in labeling medium (1 μCi of [^{3}H]inositol per 0.5 mL per well in MEM with 5% FBS). Cells were washed with MEM, 20 mM HEPES (pH 7.4), and 20 mM LiCl for 5 min at 37 °C and then treated with agonist. Total inositol phosphates were extracted and separated as previously described (*22*).

[^{35}S]GTPγS Binding Assay. Transiently transfected HEK-293 cells in 10 cm dishes were washed twice in PBS, collected with a cell scraper in ice-cold homogenization buffer [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 10 μM GDP, 1 mM PMSF, and one protease inhibitor cocktail tablet per 10 mL], and disrupted with a dounce homogenizer. Crude membranes were prepared by centrifugation at 30000*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, and 10 μM GDP]. Membranes (20 μg of protein per assay tube) were incubated in 100 pM [^{35}S]GTPγS (1250 Ci/mmol) and in the presence or absence of 100 nM or 1 μM AngII for 1 h at 30 °C. Binding was terminated by rapid filtration over GF/B filters using a cell harvester. Filters were washed three times with ice-cold dH_2O and counted with a liquid scintillation counter.

Confocal Microscopy. HEK-293 cells were transiently transfected in 35 mm confocal dishes. Cells were stimulated with 10 μM NE or 1 μM AngII and incubated at 37 °C for 15 min before being viewed. Alternatively, cells were cultured overnight at 37 °C in 10 μM phentolamine or 1 μM L158,809. Confocal microscopy was performed at 100× magnification with a Zeiss laser-scanning microscope (LSM-

510). GFP and FITC images were collected using 488 nm excitation and a 505 nm long-pass filter.

Antibody Labeling. HEK-293 cells in 35 mm confocal dishes were transiently transfected with the HA-tagged receptor in pCDNA3.1. Live cells were incubated for 1 h at 37 °C in MEM with 2% BSA and a 1:200 dilution of anti-mouse HA antibody (Cell Signaling, Beverley, MA). Cells were washed three times in MEM with 2% BSA, incubated for 20 min at 37 °C in the same buffer with a 1:200 dilution of anti-mouse IgG FITC-conjugated antibody (Sigma), and washed three times in MEM with 2% BSA before being viewed.

RESULTS

Whole Cell Binding and Receptor Expression of α_{1B}-AR R143 Mutants. Previous studies of arginine residue mutations in the DRY motif of GPCRs indicate that the mutants are expressed less well on the plasma membrane than the wild-type receptor (*3*). To compare the plasma membrane expression of the α_{1B}-ARs, whole cell binding data using [^{3}H]-prazosin are shown in Figure 1A. Scatchard plot analysis indicates that the B_{max} of the α_{1B}-AR R143E is markedly lower than that of the wild-type α_{1B}-AR. This observation was confirmed by immunofluorescence using live, unpermeabilized cells expressing the wild-type α_{1B}-AR or α_{1B}-AR R143E tagged with an HA epitope. Cells were incubated with an anti-HA antibody followed by a FITC-labeled secondary antibody. The amount of fluorescence originating from the plasma membrane of the cells expressing the α_{1B}-AR R143E (Figure 1B, right panel) is much smaller than the amount of the wild-type α_{1B}-AR (left panel), indicating a reduced level of surface expression of the α_{1B}-AR R143E. The punctate appearance of the α_{1B}-AR R143E is presumably due to the β-arrestin-bound mutant receptor clustering in clathrin-coated pits.

Signaling of α_{1B}-AR and α_{1B}-AR R143 Mutants. Relative to the wild-type receptor, a constitutively desensitized receptor should be impaired in its ability to signal through G proteins. To verify this property, the [^{3}H]IP accumulation in HEK-293 cells expressing either the wild-type α_{1B}-AR or the mutant α_{1B}-AR R143 was assessed (Figure 2). Cells expressing the wild-type α_{1B}-AR or R143K displayed a low level of accumulation of [^{3}H]IP in the absence of agonist, with an approximate 10-fold increase upon addition of 10 μM NE. In contrast, the α_{1B}-AR R143A, R143E, R143H, and R143N displayed significantly impaired ability to mediate an agonist-induced IP response.

Localization of α_{1B}-AR- and α_{1B}-AR R143-GFP Mutants in Response to NE. Since the lack of membrane expression of the α_{1B}-AR R143E shown by the binding data might suggest an impairment of receptor processing, we determined the cellular localization of the wild-type α_{1B}-AR and α_{1B}-AR R143 mutants using chimeras of the receptors tagged at the C-terminus with green fluorescent protein (GFP). Figure 3A illustrates that when HEK-293 cells are transfected with the wild-type α_{1B}-AR-GFP, the fluorescence signal as revealed by confocal microscopy originates predominantly from the plasma membrane in the absence of agonist. Addition of 10 μM NE to cells expressing the wild-type α_{1B}-AR-GFP results in a loss of plasma membrane expression and the redistribution of the receptor to endocytic vesicles.

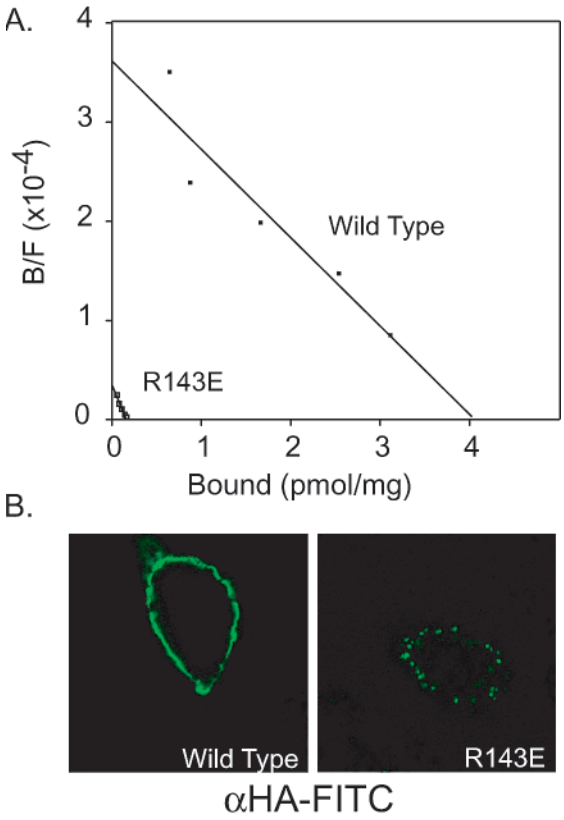

FIGURE 1: Expression of the wild-type α_{1B}-AR and α_{1B}-AR R143E in HEK-293 cells. Cells transiently transfected with cDNA for the wild-type α_{1B}-AR (■) or the α_{1B}-AR R143E mutant (□) were exposed to varying concentrations of [^{3}H]prazosin. (A) Scatchard analysis indicates that the α_{1B}-AR R143E mutants display lower B_{max} values than the wild-type α_{1B}-AR. Plasma membrane expression of the wild-type α_{1B}-AR varied between 1.5 and 4 pmol/mg of whole cell protein, while the expression of the α_{1B}-AR R143E varied between 0.2 and 0.5 pmol/mg. The K_D of the α_{1B}-AR R143E mutant for prazosin was similar to the K_D of the wild-type α_{1B}-AR for prazosin (between 1.5 and 3.5 nM). The data are representative of three independent experiments, with each point measured in duplicate. (B) Fluorescence images of live, unpermeabilized HEK-293 cells expressing the wild-type α_{1B}-AR (left) or the α_{1B}-AR R143E (right) illustrate the lower level of surface expression of the α_{1B}-AR R143E compared to that of the wild-type α_{1B}-AR. Cells were labeled with mouse monoclonal anti-HA antibody and mouse anti-IgG FITC-conjugated secondary antibody.

In contrast, Figure 3B illustrates that even in the absence of agonist the α_{1B}-AR R143-GFP mutants are localized predominantly in endocytic vesicles, similar to what was found with the constitutively desensitized V2R R137H (*8*). Exposure of cells expressing α_{1B}-AR R143-GFP mutants to agonist enhanced the endosomal distribution of the remaining plasma membrane receptor (data not shown).

Distribution of β-Arrestin-GFP in HEK-293 Cells Expressing the α_{1B}-AR. Agonist-stimulated α_{1B}-ARs have been previously shown to promote the translocation of cytosolic β-arrestin to the plasma membrane, an event that is associated with the desensitization and endocytosis of activated receptors (*23*). To observe how the distribution of β-arrestin is affected by the α_{1B}-AR R143 mutants, we coexpressed β-arrestin-GFP with these receptors in HEK-293 cells. Figure 4A illustrates that in the absence of agonist, β-arrestin-GFP is uniformly distributed in the cytosol of cells expressing the wild-type α_{1B}-AR, and that the addition of 10 μM NE to the cells results in the rapid translocation of β-arrestin-GFP

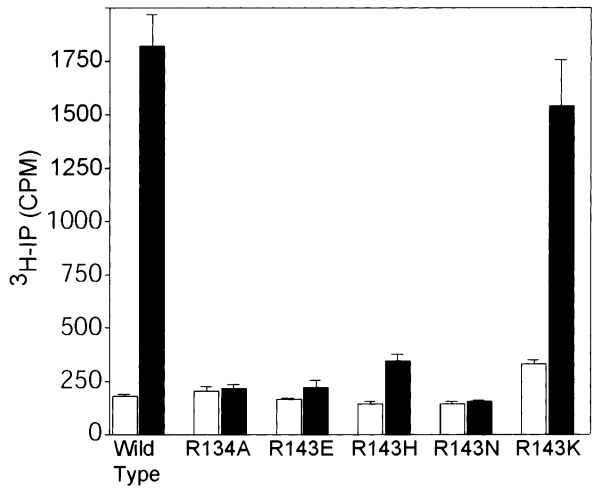

FIGURE 2: Norepinephrine (NE) stimulation of inositol phosphate accumulation. HEK-293 cells transfected with the wild-type α_{1B}-AR or the α_{1B}-AR R143 mutants were incubated for 10 min in the absence (□) or presence (■) of 10 μM NE at 37 °C. The data are representative of three independent experiments, with each point measured in triplicate.

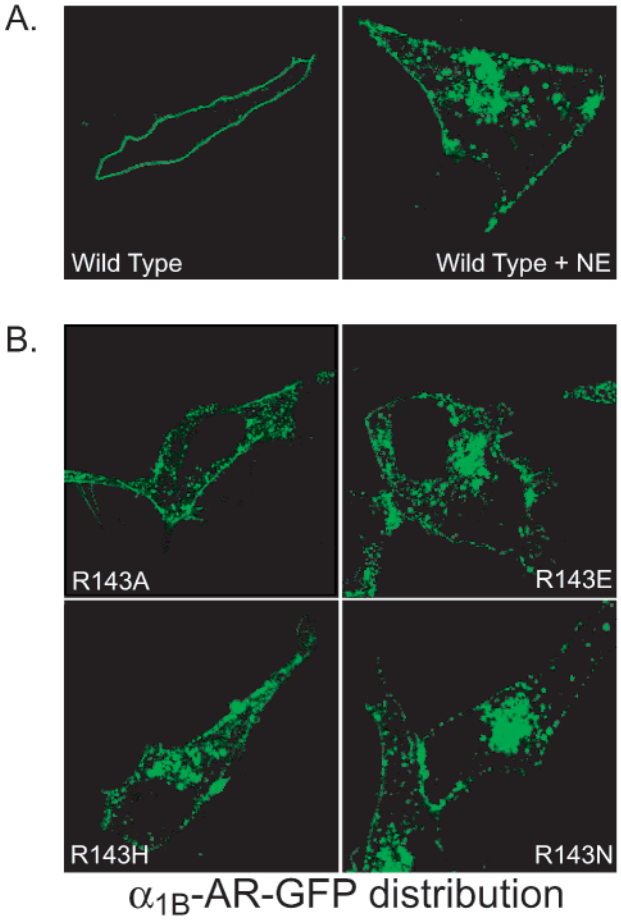

FIGURE 3: Fluorescence images of the wild-type α_{1B}-AR-GFP and the α_{1B}-AR R143-GFP mutants in HEK-293 cells. (A) Cells expressing the wild-type α_{1B}-AR-GFP in the absence of agonist (left) and upon addition of 10 μM NE (right). (B) Cells expressing the α_{1B}-AR R143-GFP mutants in the absence of agonist.

to the plasma membrane. This pattern of translocation is reminiscent of so-called class A receptors such as the β_2-adrenergic receptor (β_2-AR) (*16*). Activation of class A receptors leads to plasma membrane translocation of β-arrestin without its subsequent cotrafficking into endocytic vesicles, as is the case for class B receptors such as the V2R and $AT_{1A}R$ (*16*).

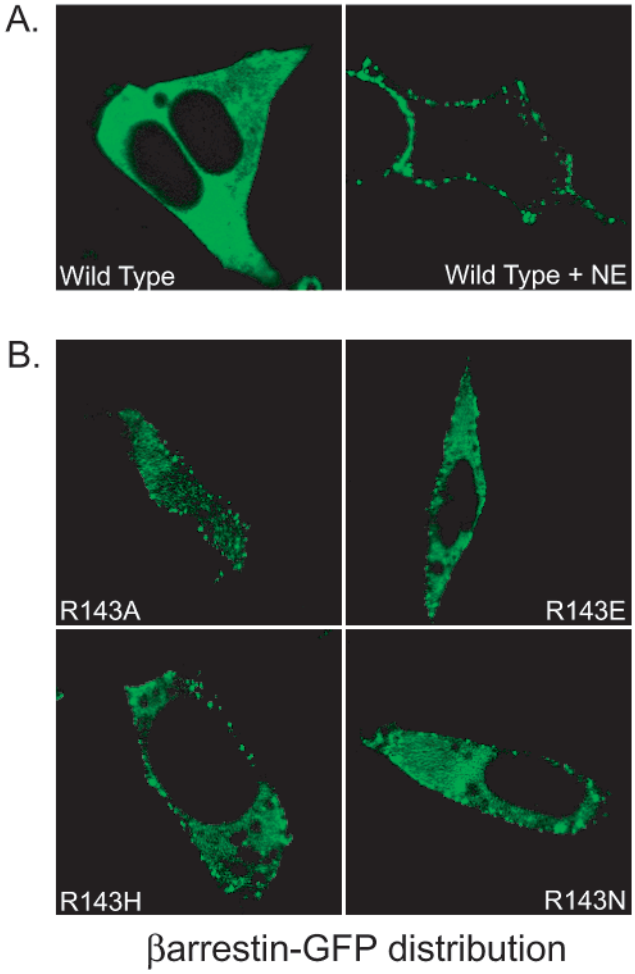

FIGURE 4: Fluorescence images of the association between β-arrestin-GFP and the wild-type α_{1B}-AR or the α_{1B}-AR R143 mutants in HEK-293 cells. (A) β-Arrestin-GFP coexpressed with the wild-type α_{1B}-AR in the absence of agonist (left) and upon addition of 10 μM agonist (right). (B) β-Arrestin-GFP coexpressed with the α_{1B}-AR R143 mutants in the absence of agonist.

In cells coexpressing β-arrestin-GFP and α_{1B}-AR R143 mutants, the localization of β-arrestin-GFP appears partially at the plasma membrane without the addition of agonist (Figure 4B). This ability of the mutants to translocate β-arrestin-GFP in the absence of agonist suggests that they have a higher affinity for β-arrestin than the wild-type receptor without agonist. The addition of 10 μM NE to cells coexpressing β-arrestin-GFP and α_{1B}-AR R143 mutants enhances the plasma membrane distribution of β-arrestin-GFP (data not shown).

Receptor and β-Arrestin Distribution in Cells Expressing the α_{1B}-AR R143K Mutant. Since the α_{1B}-AR R143K mutant appears to signal in a manner similar to that of the wild-type receptor in HEK cells [and has been reported to be constitutively active in COS cells (*3*)], we examined the localization of R143K and its β-arrestin distribution in HEK cells. Figure 5A illustrates the localization of the α_{1B}-AR R143K-GFP in endocytic vesicles in the absence of agonist, although a significant amount of receptor still appears to be visible at the plasma membrane (left panel). The addition of 10 μM NE results in a more complete localization of the

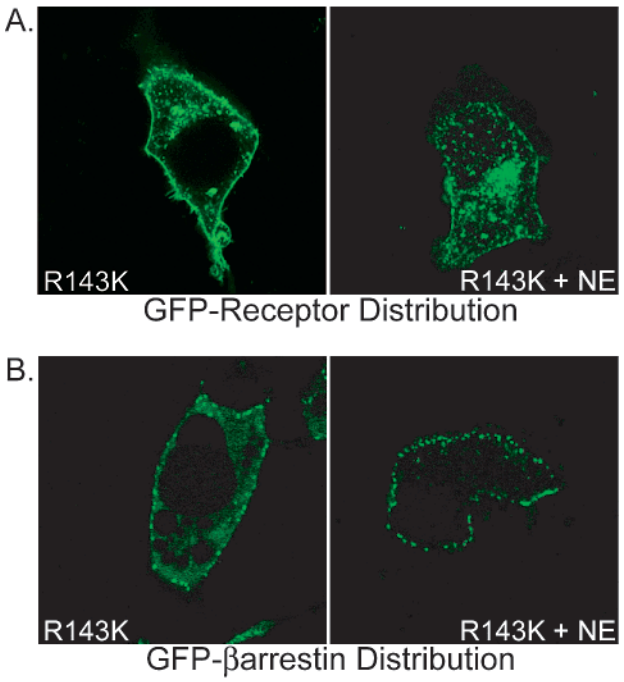

FIGURE 5: Fluorescence images of (A) the α_{1B}-AR R143K-GFP or (B) β-arrestin-GFP coexpressed with the α_{1B}-AR R143K in the absence (left) or presence (right) of 10 μM NE.

α_{1B}-AR R143K-GFP to endocytic vesicles. Figure 5B demonstrates that the α_{1B}-AR R143K is able to translocate β-arrestin-GFP to the plasma membrane in the absence of agonist (left panel), an event which is significantly enhanced upon addition of 10 μM NE (right panel).

Reversal of the Localization of the α_{1B}-AR R143 Mutant with Antagonist or K44A Dynamin. The low level of membrane expression and intracellular localization of the mutant receptors as revealed by the receptor-GFP constructs may reflect their inability to ever reach the plasma membrane. For instance, the receptors may be inappropriately folded and/or processed in the endoplasmic reticulum. However, if the loss of expression is due to their constitutive association with β-arrestin, as shown in Figure 4B, it would suggest that the mutant receptors move to the plasma membrane before being internalized into endocytic vesicles.

Two approaches were used to determine if the α_{1B}-AR R143E mutant could be trapped on the plasma membrane, reversing the phenotype of constitutive internalization in endocytic vesicles. The first method utilized phentolamine, a selective antagonist of the α_{1B}-AR. Figure 6A illustrates that HEK-293 cells transiently transfected with the wild-type α_{1B}-AR-GFP express the receptor predominantly at the plasma membrane (top left panel). HEK-293 cells transfected with the wild-type α_{1B}-AR-GFP and cultured overnight at 37 °C in the presence of 10 μM phentolamine showed no alteration in the expression of the receptor at the plasma membrane (top center panel). However, the α_{1B}-AR R143E-GFP in HEK-293 cells was predominantly localized inside endocytic vesicles (bottom left panel), but in the presence of 10 μM phentolamine, a complete reversal of phenotype was observed and the mutants were localized at the plasma membrane (bottom center panel).

The second method utilized to reverse the phenotype was the coexpression of the receptors with K44A dynamin, an endocytic protein variant that competitively inhibits the

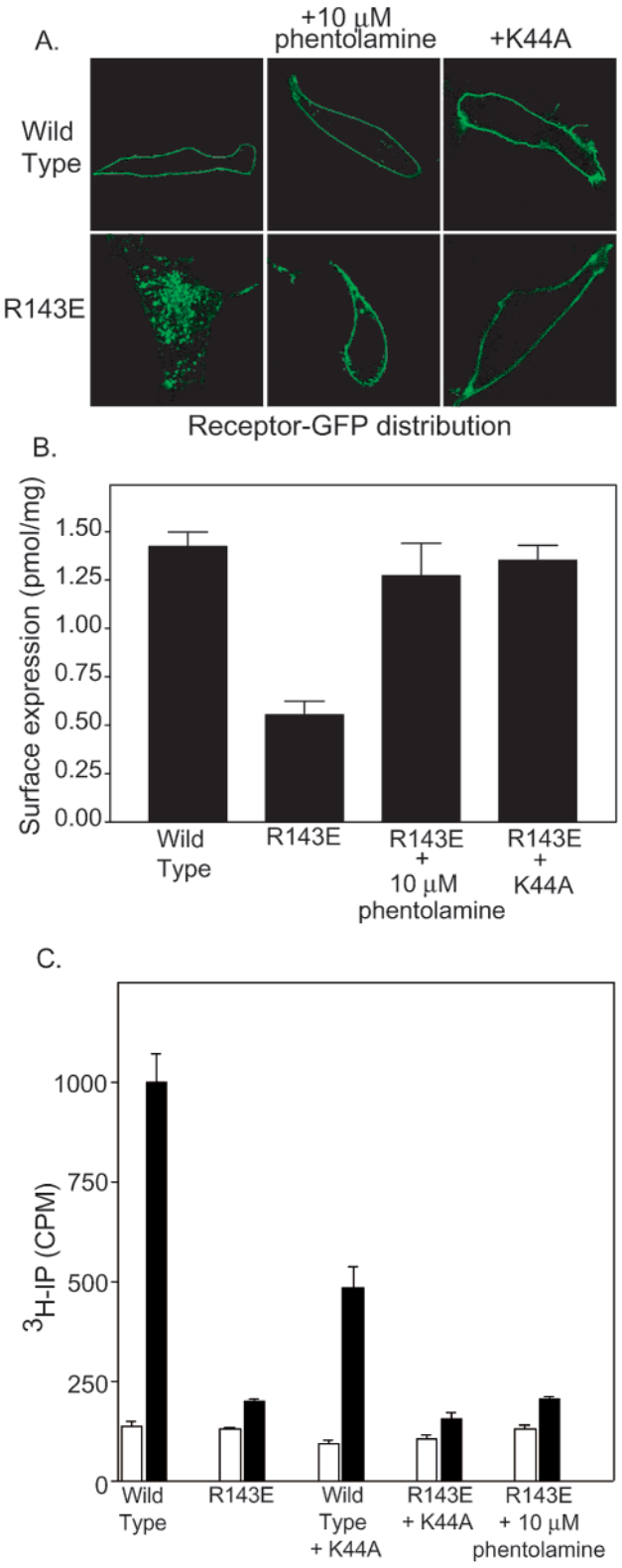

FIGURE 6: Localization, binding, and signaling properties of α_{1B}-AR in the presence of K44A dynamin or phentolamine. (A) Cells expressing the wild-type α_{1B}-AR-GFP (top) or α_{1B}-AR R143E-GFP (bottom) were left untreated (left), cultured in 10 μM phentolamine (center), or coexpressed with K44A dynamin (right). (B) Whole cell binding with 8 nM [^{3}H]prazosin on HEK-293 cells transfected with wild-type α_{1B}-AR-GFP or α_{1B}-AR R143E-GFP coexpressing K44A dynamin or cultured in 10 μM phentolamine. (C) [^{3}H]IP accumulation in cells expressing the wild-type α_{1B}-AR or α_{1B}-AR R143E either coexpressed with K44A dynamin or cultured in 10 μM phentolamine and incubated for 10 min in the absence (□) or presence (■) of 10 μM NE.

fission of clathrin-coated vesicles from the plasma membrane (*17*). The α_{1B}-AR R143E-GFP when coexpressed with K44A dynamin was unable to undergo endocytosis, and its expression remained at the plasma membrane (Figure 6A, bottom right panel).

Whole cell binding (Figure 6B) confirmed that while the untreated α_{1B}-AR R143E cells displayed a lower level of receptor expression on the plasma membrane than the wild-type α_{1B}-AR cells, the presence of an antagonist or the coexpression of K44A dynamin was able to increase the level of expression of the α_{1B}-AR R143E on the plasma membrane to the level of the wild-type α_{1B}-AR. The coexpression of K44A dynamin in cells with the α_{1B}-AR R143E, however, failed to reestablish signaling of the β-arrestin-associated receptor, as did culturing the cells in 10 μM phentolamine (Figure 6C). The level of [^{3}H]IP accumulation in HEK-293 cells coexpressing the wild-type α_{1B}-AR with K44A dynamin and stimulated with 10 μM NE is reduced by ~2-fold compared to that in cells expressing only the wild-type α_{1B}-AR. This reduction in the level of signaling of the wild-type receptor coexpressed with K44A dynamin is presumably because the receptors are only able to signal once before becoming trapped in clathrin-coated pits that are unable to recycle the receptors back to the membrane. The level of [^{3}H]IP accumulation in HEK-293 cells coexpressing the α_{1B}-AR R143E with K44A dynamin and stimulated with 10 μM NE, however, is equal to the level of signaling observed in cells expressing only the α_{1B}-AR R143E mutant. It would seem likely that the level of signaling in the presence of K44A dynamin is not increased because the receptors are already desensitized by β-arrestin prior to the addition of agonist and are now trapped at the plasma membrane rather than in vesicles. The fact that culturing the cells expressing the α_{1B}-AR R143E mutant in 10 μM phentolamine reverses the receptor localization back to the plasma membrane without reestablishing signaling properties may indicate that the conformation of the receptor in the absence of bound β-arrestin still renders the mutant unable to signal. It is also possible that the time it takes during the experiment to wash the cells of antagonist and stimulate them with agonist is sufficient for β-arrestin to reassociate with the mutant receptor and abrogate its ability to signal.

Receptor Coupling and Distribution of β-Arrestin in HEK-293 Cells Expressing the $AT_{1A}R$. To determine if the phenotype of constitutive desensitization resulting from mutation of the DRY motif arginine could be extended to another GPCR, we repeated the receptor-GFP localization and β-arrestin-GFP translocation experiments using the $AT_{1A}R$. Figure 7A illustrates that the wild-type $AT_{1A}R$-GFP when transfected into HEK-293 cells is expressed on the plasma membrane, and that addition of 1 μM AngII results in the complete internalization of the receptor into endocytic vesicles (top panels). In contrast, the $AT_{1A}R$ R126H-GFP transfected into HEK-293 cells is localized in endocytic vesicles without the addition of agonist, although a significant portion of the receptor still appears to be visible at the plasma membrane (bottom left panel). The cells expressing the $AT_{1A}R$ R126H-GFP redistribute the receptors localized in vesicles to the plasma membrane in the presence of 1 μM L158,809, an $AT_{1A}R$ selective antagonist (bottom right panel). Figure 7B shows that β-arrestin-GFP is uniformly distributed in the cytosol of HEK-293 cells expressing the

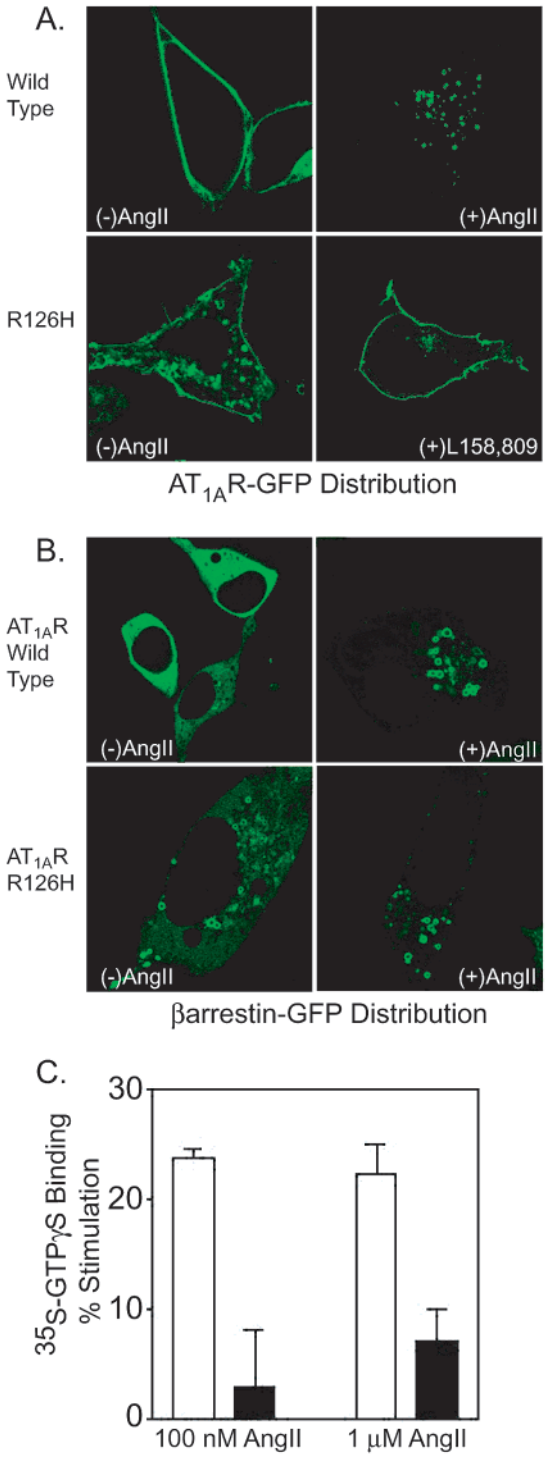

FIGURE 7: G protein coupling, localization, and β-arrestin-GFP distribution of the $AT_{1A}R$. (A) Cells expressing the wild-type $AT_{1A}R$-GFP in the absence of agonist (top left) and upon the addition of 1 μM AngII (top right). Cells expressing the $AT_{1A}R$ R126H-GFP in the absence of agonist (bottom left) and cultured in 1 μM L158,809 (bottom right). (B) β-Arrestin-GFP coexpressed with the wild-type $AT_{1A}R$ in the absence of agonist (top left) and upon the addition of 1 μM AngII (top right). β-Arrestin-GFP coexpressed with the $AT_{1A}R$ R126H in the absence of agonist (bottom left) and upon the addition of 1 μM AngII (bottom right). (C) [^{35}S]GTPγS coupling assessed in membranes prepared from HEK-293 cells transiently transfected with the wild-type $AT_{1A}R$ (□) or $AT_{1A}R$ R126H (■) and $G\alpha_q$ protein and incubated in either 100 nM or 1 μM AngII for 1 h at 30 °C. The data are representative of three independent experiments, with each point measured in triplicate.

wild-type $AT_{1A}R$, and that addition of 1 μM AngII causes redistribution of the β-arrestin-GFP into endocytic vesicles (top panels). In contrast, β-arrestin-GFP when coexpressed with the $AT_{1A}R$ R126H is distributed predominantly in endocytic vesicles in the absence of agonist (bottom left panel). Addition of 1 μM AngII to cells expressing the $AT_{1A}R$ R126H results in a complete translocation of β-arrestin-GFP to endocytic vesicles (bottom right panel).

While several of the α_{1B}-AR R143 mutants are severely impaired in agonist-mediated IP_3 production, a similar approach with the $AT_{1A}R$ R126H revealed that this mutant was still able to retain signaling activity. Under our experimental conditions in HEK cells transfected with the $AT_{1A}R$, the relationship between receptor expression and signaling activity was nonlinear. Therefore, to assess the differences in G protein coupling ability between the wild-type $AT_{1A}R$ and $AT_{1A}R$ R126H, it was necessary to utilize a [^{35}S]GTPγS binding assay which is the most proximal measure of receptor coupling activity and does not rely on an amplified secondary messenger signal such as IP_3. Figure 7C shows that HEK cells transiently transfected with $G\alpha_q$ and the wild-type $AT_{1A}R$ when stimulated with 100 nM or 1 μM AngII display a significant increase in the level of [^{35}S]GTPγS binding over basal levels. In contrast, the $AT_{1A}R$ R126H exhibited a much lower level of coupling when stimulated with 100 nM or 1 μM AngII than the wild-type $AT_{1A}R$. This observation was made in spite of the fact that the level of expression of the $AT_{1A}R$ R126H when measured by whole cell binding with [^{3}H]angiotensin was found to be almost 2-fold higher than that of the wild-type $AT_{1A}R$ (wild-type $AT_{1A}R$, $B_{max} = 115.2 \pm 21.4$ fmol/mg of protein; $AT_{1A}R$ R126H, $B_{max} = 213 \pm 22.5$ fmol/mg of protein). One possible explanation for this result may be that the constitutive association of β-arrestin with the $AT_{1A}R$ R126H prevents the receptor from entering a degradation pathway. This explanation is consistent with the previous observation that the $AT_{1A}R$ upon agonist-stimulated endocytosis may recycle to the plasma membrane or be targeted for lysosomal degradation (*24*). Another possibility that cannot be excluded is that in contrast to the α_{1B}-AR antagonist [^{3}H]prazosin, the agonist [^{3}H]angiotensin is able to label internalized $AT_{1A}Rs$.

Signaling of the $AT_{1A}R$ R126H and Its Association with β-Arrestin in the Presence of GRKs. Given the fact that other GPCRs with DRY motif arginine mutations lose their ability to couple to G proteins and that the $AT_{1A}R$ R126H is able to translocate β-arrestin-GFP in the absence of agonist (as shown in Figure 7B), we speculated that perhaps the $AT_{1A}R$ R126H would also exhibit a loss of signaling phenotype. However, HEK cells transfected with the $AT_{1A}R$ R126H and stimulated with AngII are able to accumulate [^{3}H]IP at levels identical to those of HEK cells transfected with the wild-type $AT_{1A}R$ and stimulated with AngII (data not shown).

Our previous studies with the V2R R137H suggested a possible explanation as to why the $AT_{1A}R$ R126H retains signaling ability. The V2R R137H is unable to activate cyclic AMP, whereas a mutant V2R R137H Ala6, with the C-terminal GRK phosphorylation sites removed, had its signaling ability partially restored (*8*). Therefore, we hypothesized that increasing the cellular GRK activity would intensify the desensitization phenotype of the $AT_{1A}R$ R126H. To determine what effect the expression of GRKs in HEK cells expressing the $AT_{1A}R$ R126H has on the distribution of β-arrestin, cells were transfected for confocal viewing with β-arrestin-GFP, wild-type $AT_{1A}R$, or $AT_{1A}R$ R126H, and either with or without the cotransfection of GRK2. Figure 8A (left top and bottom panels) shows that in HEK cells transfected with β-arrestin-GFP and the wild-type $AT_{1A}R$, the localization of β-arrestin-GFP is distributed throughout the cytosol with or without the cotransfection of GRKs. Upon addition of 1 μM AngII to these cells, the β-arrestin-GFP translocates to endocytic vesicles (Figure 8A, right top and bottom panels). Figure 8B shows that HEK cells transfected with β-arrestin-GFP and the $AT_{1A}R$ R126H in the absence of agonist result in a partial translocation of β-arrestin-GFP to endocytic vesicles (top left panel), with a notable amount of β-arrestin-GFP remaining in the cytosol. This remaining cytosolic β-arrestin-GFP redistributes to endocytic vesicles upon the addition of 1 μM AngII, as shown in Figure 8B (top right panel). HEK cells transfected with β-arrestin-GFP, the $AT_{1A}R$ R126H, and GRK2, however, result in a complete translocation of β-arrestin-GFP to endocytic vesicles without the addition of agonist as shown in Figure 8B (bottom left panel). The β-arrestin-GFP remains completely localized in endocytic vesicles when these cells are exposed to 1 μM AngII (Figure 8B, bottom right panel).

The complete redistribution of β-arrestin-GFP to endocytic vesicles in cells expressing the $AT_{1A}R$ R126H and GRK suggests that the increased GRK activity results in an enhanced state of receptor desensitization. To demonstrate the loss of receptor expression at the plasma membrane due to desensitization, we employed a whole cell binding assay with 12 nM [^{3}H]angiotensin to assess the expression of the $AT_{1A}R$ receptors on cells either transfected with the wild-type $AT_{1A}R$ or $AT_{1A}R$ R126H alone, or cotransfected with GRK and β-arrestin. Figure 8C shows that cells expressing the wild-type $AT_{1A}R$ show little difference in their plasma membrane expression of the receptor when cotransfected with GRK and β-arrestin. However, a significant decrease in the level of plasma membrane expression of the $AT_{1A}R$ R126H was evident when cells were cotransfected with GRK and β-arrestin, suggesting that the increased GRK activity results in an enhanced state of receptor desensitization and endocytosis. This is consistent with the observation that HEK cells cotransfected with the $AT_{1A}R$ R126H and GRK accumulate IP in response to AngII at lower levels than cells cotransfected with the wild-type receptor and GRK (data not shown).

DISCUSSION

In this paper, we provide direct evidence that the paradigm of constitutive desensitization previously elucidated for a naturally occurring loss-of-function mutant of the V2R (*8*) can be extended to other members of the GPCR family carrying analogous mutations. Several mutations of the highly conserved arginine residue of the DRY motif, which in the V2R R137H produces a constitutively desensitized phenotype, have been reported as loss-of-function mutants in the α_{1B}-AR (*3*). Similar to the V2R R137H phenotype, the α_{1B}-AR R143A, R143E, R143H, and R143N mutants are decoupled from G proteins and display decreased expression at the plasma membrane. While the α_{1B}-AR R143K retains its ability to accumulate IP in response to agonist, it also appears to be partially localized in endocytic vesicles and to translocate β-arrestin in the absence of agonist. In terms of IP accumulation, the fact that the α_{1B}-AR R143K does

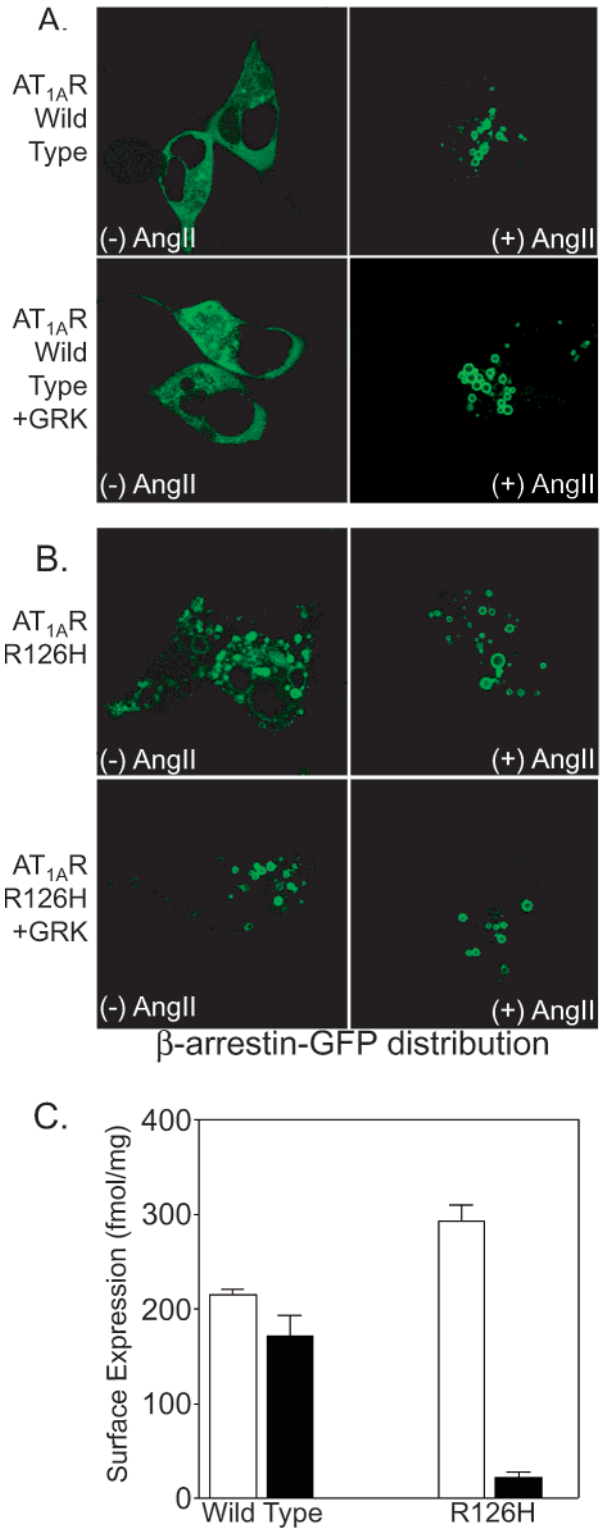

FIGURE 8: Fluorescence images of β-arrestin-GFP in HEK cells expressing the $AT_{1A}R$ in the presence of GRK. (A) β-Arrestin-GFP distribution in cells expressing the wild-type $AT_{1A}R$ (top panels) and cells expressing the wild-type $AT_{1A}R$ and GRKs (bottom panels) when exposed to 1 μM AngII (top and bottom right). (B) β-Arrestin-GFP distribution in cells expressing the $AT_{1A}R$ R126H (top left) and upon addition of 1 μM AngII (top right). β-Arrestin-GFP distribution in cells expressing the $AT_{1A}R$ R126H and GRKs (bottom left) and upon addition of 1 μM AngII (bottom right). (C) Whole cell binding with 12 nM ^{3}[H]angiotensin on cells transfected with either the wild-type $AT_{1A}R$ or $AT_{1A}R$ R126H alone (□), or cotransfected with β-arrestin and GRKs (■).

not behave as a constitutively active receptor in HEK-293 cells as it does in COS cells (*3*) most likely reflects the substantial difference in the levels of endogenous β-arrestin in the two cell types. Since it has been demonstrated that HEK-293 cells express more than 3-fold higher levels of endogenous β-arrestin than COS cells (*25*), it is possible that the threshold of β-arrestins required to silence a constitutively active receptor is achievable in HEK-293 cells, but not in COS cells.

Although substitution of the entire DRY motif in the $AT_{1A}R$ has been reported to result in complete signaling impairment of the receptor (*15*), substitution of the arginine for histidine alone does not affect its inherent signaling ability as assessed by IP accumulation. However, the $AT_{1A}R$ R126H exhibits a marked reduction in its ability to couple to $G\alpha_q$ proteins as assessed by [^{35}S]GTPγS binding, localizes to endocytic vesicles, and is associated with β-arrestin in the absence of agonist. The observation that in the absence of agonist both the $AT_{1A}R$ R126H-GFP and the α_{1B}-AR R143K-GFP remain partially at the plasma membrane despite a portion of these receptors being internalized may be an explanation for why these particular mutants retain the ability to induce an agonist-mediated IP response. In all cases, however, the receptor phenotypes support our hypothesis that substitution of the arginine residue in the DRY motif results in constitutive desensitization that may or may not be accompanied by a complete loss of receptor function as assessed by secondary messengers.

The vesicular localization of the α_{1B}-AR and $AT_{1A}R$ arginine mutants is completely redistributed to the plasma membrane by the addition of selective antagonists or dominant negative K44A dynamin. These findings suggest that these mutant receptors are properly folded and initially targeted to the plasma membrane. The mechanism by which the K44A dynamin mutant inhibits agonist-mediated GPCR internalization has been previously characterized (*16*). As receptor−β-arrestin complexes are targeted to clathrin-coated pits, K44A dynamin inhibits the fission of the clathrin-coated vesicles from the plasma membrane, thus preventing internalization of the constitutively desensitized receptors (*8*, *9*). The mechanism by which the antagonists phentolamine and L158,809 prevent internalization of the mutant receptors, however, may have interesting implications for other potential GPCR mutants with this phenotype. Extracellular antagonists, by locking the receptors into their inactive form, may prevent the receptors from being recognized by the GRK, binding β-arrestin, and being constitutively moved to intracellular compartments. An alternate explanation that cannot be excluded is that prolonged exposure of cells to the antagonists could provide access of the compounds to the intracellular receptor−β-arrestin complexes and promote complex dissociation, thus helping the mutant receptors to move back to the plasma membrane. A different mechanism for explaining how intracellular antagonists could increase the level of plasma membrane complement of V2R mutants associated with nephrogenic diabetes insipidus has been proposed (*26*). In this case though, the authors suggest that the antagonists provide a chaperoning function to incorrectly folded mutant receptors (*26*).

The degree of conservation of the DRY motif arginine suggests that the constitutively desensitized phenotype observed in the V2R R137H might be extended to other GPCRs. In agreement with this, we found that the arginine mutation in the DRY motif of two distinct receptors, the α_{1B}-

AR and $AT_{1A}R$, produced phenotypes similar to the V2R R137H. Additionally, we examined a close family member of the α_{1B}-AR, the β_2-adrenergic receptor (β_2-AR). A GFP-tagged β_2-AR R131H mutant also displayed a phenotype of constitutive internalization that was similar to the α_{1B}-AR R143 mutants (data not shown). A previous study has indicated that the β_2-AR R131H when expressed in L*tk*$^-$ murine cells still retains the ability to signal through G proteins, although it should be noted that the experiment utilized to assess the receptor's signaling ability was the quantification of the second messenger cAMP (*27*). Therefore, there appears to be varying degrees of signaling activity within the constitutively desensitized phenotype. Arginine substitutions in the DRY motif result in a complete loss of signaling activity in the V2R R137H, several α_{1B}-AR mutants, and potentially other receptors that have been reported to exhibit a loss-of-function phenotype. The arginine-substituted $AT_{1A}R$ and β_2-AR, and specifically the α_{1B}-AR R143K, despite showing properties of constitutive desensitization, still retain some signaling ability. As it has been shown that increasing the levels of the cellular complements of GRK and β-arrestin enhances receptor desensitization, it may be that the levels of expression of these proteins are the ultimate arbiters in determining the desensitization state of a given receptor.

This emerging paradigm of constitutive receptor desensitization may represent an important step in our understanding of the mechanisms underlying GPCR signaling in certain receptor-related pathophysiologies. It is possible that other examples of naturally occurring mutations leading to a phenotype of hormonal resistance might exist. The elucidation of molecular determinants involved in constitutive desensitization such as association with GRK, β-arrestin, and intracellular trafficking of the receptor−β-arrestin complexes should help in identifying other examples of this phenotype.

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