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Helix I of β-Arrestin Is Involved in Postendocytic Trafficking but Is Not Required for Membrane Translocation, Receptor Binding, and Internalization

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ABSTRACT

β-arrestins bind to phosphorylated, seven-transmembrane-spanning, G protein-coupled receptors (GPCRs), including the type 1 angiotensin II receptor (AT_1R), to promote receptor desensitization and internalization. The AT_1R is a class B GPCR that recruits both β-arrestin1 and β-arrestin2, forming stable complexes that cotraffic to deep-core endocytic vesicles. β-Arrestins contain one amphipathic and potentially amphitropic (membrane-targeting) α-helix (helix I) that may promote translocation to the membrane or influence receptor internalization or trafficking. Here, we investigated the trafficking and function of β-arrestin1 and β-arrestin2 mutants bearing substitutions in both the hydrophobic and positively charged faces of helix I. The level of expression of these mutants and their cytoplasmic localization (in the absence of receptor activation) was similar to wild-type β-arrestins. After angiotensin II stimulation, both wild-type and β-arrestin mutants translocated to the cell membrane, although recruitment was weaker for mutants of the hydrophobic face of helix I. For all β-arrestin mutants, the formation of deep-core vesicles was less observed compared with wild-type β-arrestins. Furthermore, helix I conjugated to green fluorescent protein is not membrane-localized, suggesting that helix I, in isolation, is not amphitropic. Bioluminescence resonance energy transfer analysis revealed that both wild-type and β-arrestin mutants retained a capacity to interact with the AT_1R, although the interaction with the mutants was less stable. Finally, wild-type and mutant β-arrestins fully supported receptor internalization in human embryonic kidney cells and mouse embryonic fibroblasts deficient in β-arrestin1 and -2. Thus, helix I is implicated in postmembrane trafficking but is not strongly amphitropic.

The nonvisual arrestins, β-arrestin1 (βarr1) and β-arrestin2 (βarr2), are ubiquitously expressed and regulate the activity of hundreds of GPCRs, including the type 1 angiotensin II (AngII) receptor (AT_1R). β-Arrestins bind to activated and phosphorylated GPCRs, promoting receptor internalization and preventing further interaction of receptors with G proteins, thereby attenuating initial signaling. They can also function as scaffolds to recruit additional signaling/regulatory molecules to the receptor (Luttrell et al., 1999; DeFea et al., 2000; Hall and Lefkowitz, 2002). The recruitment and trafficking of β-arrestins can be visualized using confocal microscopy of green fluorescent protein (GFP)-labeled βarr1 and -2. Derived from preferential trafficking of βarr1 and -2, Oakley et al. (2000) proposed the classification of GPCRs as either class A or class B—the AT_1R is a class B GPCR that recruits both βarr1 and -2 with equal affinity and forms stable complexes that internalize via clathrin-coated pits into deep-core endocytic vesicles. In contrast, class A GPCRs, such as the β_2-adrenergic receptor (β_2AR), interact transiently and preferentially with βarr2 and dissociate from it soon after receptor internalization.

βarr1 (418 amino acids) shares 78% sequence homology with βarr2 (410 amino acids). The crystal structure of arrestin in its

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ABBREVIATIONS: βarr, β-arrestin; GPCR, G protein-coupled receptor; AngII, angiotensin II; AT_1R, type 1 angiotensin II receptor; GFP, green fluorescent protein; β_2AR, β_2-adrenergic receptor; RGS, regulators of G protein signaling; GRK, G protein-coupled receptor kinase; Rluc, *Renilla reniformis* luciferase; TRHR1, thyrotropin-releasing hormone receptor 1; MEF, mouse embryonic fibroblast; BRET, bioluminescence resonance energy transfer; PCR, polymerase chain reaction; HEK, human embryonic kidney; EGFP, enhanced green fluorescent protein; βarr1/2KO, β-arrestin1/2 knockout.

resting state (Han et al., 2001) reveals N and C domains flanking a polar core. Elements within both domains have been shown to bind phosphorylated and activated GPCRs (Gurevich and Gurevich, 2004; Vishnivetskiy et al., 2004). Although the structural changes that underpin arrestin activation are poorly understood, it is predicted that this process probably involves disruption of a key salt bridge in the polar core of arrestin by phosphorylated amino acids in the receptor and subsequent destabilization of the arrestin structure to then accommodate the cytoplasmic face of the receptor (Han et al., 2001; Gurevich and Gurevich, 2004). A novel feature of this activation is the proposed release of an α-helix in the N domain (helix I), which is highly conserved in βarr1 (T^{98}RLQERLIKKL^{108}) and βarr2 (T^{99}RLQDRLLKKL^{109}). In the basal state of β-arrestin, the hydrophobic face of helix I is completely buried in the hydrophobic cage formed by the β-strands of the N domain and the carboxyl terminus. Activation of β-arrestin is believed to destabilize the constraining hydrophobic pocket, allowing helix I to swing from its resting position and insert within the membrane bilayer and strengthen the receptor-arrestin complex (Han et al., 2001). Helix I is amphipathic and potentially amphitropic (membrane-targeting), with hydrophobic residues (Leu100, Leu104, and Leu108, βarr1 numbering) aligned on one side of the helix and positive charges (Arg99, Arg103, Lys106, and Lys107) aligned on the opposite side (Han et al., 2001). This suggests a possible role in membrane targeting and facilitation of receptor internalization and trafficking. However, there is currently no evidence to support this directly.

Various studies have shown that many intracellular signaling and regulatory proteins (e.g., the Ras family of GTPases and Src tyrosine kinases) use amphitropism to reversibly attach to the cell membrane during activation (Johnson and Cornell, 1999). Recent studies have revealed that components of the GPCR activation/deactivation cycle also seem to use amphitropism to modulate function. For example, helix VIII in the proximal carboxyl terminus of rhodopsin, angiotensin, and oxytocin receptors is a membrane-based sensor (Krishna et al., 2002; Mozsolits et al., 2002; Zhong et al., 2004). The basically charged, amphipathic α-helices in the regulators of G protein signaling (RGS) proteins and GPCR kinases (GRKs) also promote reversible membrane association (Chen et al., 1999; Bernstein et al., 2000; Thiyagarajan et al., 2004). The hydrophobic face of the α-helix is believed to intercalate into the lipid bilayer, whereas the positively charged amino acids on the opposing face interact with the head groups of anionic phospholipids.

In this study, we investigated the role of helix I in β-arrestin trafficking, receptor interaction, and function. We observed that mutations to helix I that negate the hydrophobic and charged aspects do not abrogate membrane recruitment of arrestins in response to receptor activation. It is interesting that these mutants retain a capacity to interact with activated receptors and to fully support receptor internalization, yet they do not traffic efficiently to deep-core endocytic vesicles.

Materials and Methods

Materials. Anti-GFP polyclonal antibody was purchased from BD Biosciences Clontech (Palo Alto, CA), and AngII was from Auspep (Melbourne, Australia). The SuperSignal West Pico Chemiluminescent was purchased from Pierce Chemical (Rockford, IL). Radiolabeled [^{125}I]AngII (specific activity >2000 Ci/mmol) was provided by ProSearch (Melbourne, Australia). All other chemicals were from Sigma-Aldrich (St. Louis, MO) or BDH Laboratory Supplies (Poole, Dorset, UK).

Plasmids. The K-ras-GFP construct (referred to as tK-GFP) was provided by J. Hancock (Department of Pathology, University of Queensland, Brisbane, Australia). β_2AR plasmid was provided by R. Summers (Department of Pharmacology, Monash University, Melbourne, Australia). The construction of hemagglutinin epitope-tagged wild-type $AT_{1A}R$, an AT_1R-EGFP, and the dominant-negative βarr1 (βarr1$^{318–419}$) have been reported previously (Thomas et al., 1998; Qian et al., 2001; Holloway et al., 2002). A thyrotropin-releasing hormone receptor (TRHR1)-*Renilla reniformis* luciferase (Rluc) construct was described previously (Hanyaloglu et al., 2002) and the AT_1R-Rluc construct was generated by amplifying the hemagglutinin epitope AT_1R using PCR (sense primer, T7 primer; antisense primer, 5′-GAA***GCGGCCG***CTCCACCTCAAAACAAGACGCAGG) with an extra NotI site (boldface italic) and subsequently subcloning into pcDNA3/Rluc.

GFP and HcRed versions of the β-arrestin mutants were constructed using PCR-based site-directed mutagenesis (ExSite; Stratagene, La Jolla, CA) from βarr1-GFP, βarr2-GFP, and βarr2-HcRed (kindly provided by M. G. Caron, Duke University Medical Center, Durham, NC). LLL/A mutant versions of βarr1-GFP and βarr2-GFP contained three substitutions of leucine residues with alanine (βarr1: Leu99, Leu103, and Leu107; βarr2: Leu100, Leu104, and Leu108), whereas RRKK/Q mutant versions of βarr1-GFP and βarr2-GFP are characterized by quadruple substitutions of arginine and lysine residues with glutamine (βarr1: Arg98, Arg102, Lys105, and Lys106; βarr2: Arg99, Arg103, Lys106, and Lys107). 5′-Oligonucleotides used for mutagenesis were (5′-3′): βarr1LLL/A, ***CGG***GCCATCAAGAAGGCGGGCGAGCATGCCTACCCC (sense) and *CTC*TTGTGCCCGAGTCAGTGGCTTCTTGTC (antisense); βarr2LLL/A, ***GCC***CTGAAGAAGGCGGGCCAGCATGCCCACCCC (sense) and *CCG*GTCCTGTGCGCGGGTGGGGGGCCGAGG (antisense); βarr1RRKK/Q, ***CTG***ATCCAGCAGCTGGGCGAGCATGCCTACCCC (sense) and ***CTG***CTCTTGTAGCTGAGTCAGTGGCTTCTTGTC (antisense); and βarr2RRKK/Q, ***CTG***CTGCAGCAGTTGGGCCAGCATGCCCACCCC (sense) and ***CTG***GTCCTGTAGCTGGGTGGGGGGCCGAGGTGG (antisense).

Oligonucleotides were 5′-phosphorylated using T4 polynucleotide kinase. Silent restriction sites (BsrBI, DraII, or PvuII) were introduced to facilitate the screening of mutated clones (boldface italic) formed after ligation of PCR product created by βarr1LLL/A, βarr2LLL/A, βarr1RRKK/Q, or βarr2RRKK/Q primers, respectively.

Expression plasmids for GFP-helix I were constructed by ligating HindIII/BamHI cDNA fragments of βarr1 and -2, corresponding to helix I, into the cloning site of pEGFP-C1 (BD Biosciences Clontech). GFP-βarr1helix ended with TRLQERLIKKL, and GFP-βarr2helix terminated with TRLQDRLLKKL. All constructs were verified by sequencing.

Cell Culture and Transfection. Mouse embryonic fibroblasts (MEFs; provided by R. J. Lefkowitz, Duke University Medical Center, Durham, NC) from a βarr1/2 knockout (βarr1/2KO) (Kohout et al., 2001) and HEK-293 (American Type Culture Collection, Manassas, VA) cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, and 0.1 mg/ml streptomycin/penicillin (Invitrogen, Melbourne, Australia). For receptor expression and internalization studies, cells were grown to 60 to 80% confluence on 12-well culture plates. Cells were transiently cotransfected with 0.3 μg DNA/well wild-type AT_1R with or without 0.3 μg DNA/well of either βarr1-GFP, βarr2-GFP, or mutants and various amount of pRc/CMV to a total of 0.6 μg DNA/well using LipofectAMINE (Invitrogen). For confocal experiments, cells were plated on collagen-coated 35-mm glass-bottom dishes (MatTek, Ashland, MA) and transiently cotransfected with 30 ng DNA of either wild-type βarr1-GFP, βarr2-GFP, βarr2-HcRed, or mutants with either 70 ng of DNA of AT_1R or β_2AR (or 10 ng of AT_1R-EGFP) and various amount of pRc/CMV to a total of 0.3 μg DNA/dish, using

LipofectAMINE 2000 (Invitrogen). Cells were assayed 24 to 48 h after transfection.

Confocal Microscopy. HEK-293 cells were changed into serum-free Dulbecco's modified Eagle's medium at least 2 h before commencing confocal experiments. Localization and trafficking of β−arrestins in response to AngII (100 nM) or isoproterenol (10 μM) stimulation was viewed with a 63 × 1.2 numerical aperture water-immersion objective on a heated stage at 37°C. Images were collected using a Zeiss LSM 510 META confocal microscope (Carl Zeiss Inc., Thornwood, NY). To compare trafficking between wild-type and mutant β-arrestins after 60 min of AngII stimulation, we randomly chose five fields (10 cells per field) and scored whether β-arrestins had trafficked into vesicles or were retained at the cell surface. Values are expressed as a percentage of the total number of cells examined.

Bioluminescence Resonance Energy Transfer Assay. β-arrestin-AT_1R interaction in living cells was examined using bioluminescence resonance energy transfer (BRET) as described previously (Kroeger et al., 2001; Hanyaloglu et al., 2002). In brief, AT_1R-Rluc and either wild-type βarr1-GFP, βarr2-GFP, or mutants were coexpressed in COS-7 cells (American Type Culture Collection). The transfected cells were stimulated with AngII, and readings were measured at 0 and 10 min in the presence of the substrate of *R. reniformis* luciferase, coelenterazine (Molecular Probes, Eugene, OR). In addition, a time-course study was performed in which cells were incubated in the presence of a long-acting substrate, EnduRen (Promega, Madison, WI). Thereafter, cells were stimulated with AngII, and consecutive readings were collected for 60 min. The binding of βarr1-GFP and βarr2-GFP to the AT_1R-Rluc was measured as changes in the BRET ratio calculated using the equation 515 nm/475 nm) − (515 nm/475 nm for Rluc alone). Measurements were performed at the wavelengths of 475 and 515 nm. An increased ratio indicates β-arrestin-AT_1R interaction.

AT_1R Internalization. Receptor internalization assays were determined as acid-insensitive [^{125}I]AngII receptor binding as described previously (Thomas et al., 1995).

Results

Trafficking of βarr1 and -2 Helix I Mutants Using Confocal Microscopy. To examine the role of helix I in arrestin function, we constructed various β-arrestin mutants (Fig. 1) in which hydrophobic residues on one face of the helix were substituted to alanine (βarr1LLL/A and βarr2LLL/A) and in which positively charged amino acids were replaced with glutamine (βarr1RRKK/Q and βarr2RRKK/Q). Before investigating the function of these β-arrestin mutants, we confirmed that all constructs expressed at equivalent levels in HEK-293 cells (Fig. 2).

The AT_1R is a class B GPCR in that it recruits and binds both βarr1 and βarr2 and forms stable complexes that traffic to deep-core endocytic vesicles. As expected, wild-type βarr1-GFP rapidly translocated from the cytoplasm to the cell surface (5 min) and then redistributed into deep-core endocytic vesicles (60 min) after AngII-induced AT_1R stimulation (Fig. 3A). In addition, we randomly chose five fields (10 cells per field) and scored whether wild-type or β-arrestin mutants trafficked into vesicles or were retained at the cell surface; for the wild-type βarr1-GFP, after 60 min of AngII stimulation, all 50 cells examined trafficked into deep-core vesicles. It is interesting that the βarr1LLL/A-GFP construct, bearing substitutions within the hydrophobic face, translocated to the cell membrane, and although some pit formation occurred with longer AngII stimulation, the majority was localized to the juxta-membrane region (74% of cells were recruited to the cell membrane versus 26% that were trafficked to vesicles). The βarr1RRKK/Q mutant, bearing substitutions within the positively charged residues, also maintained the capacity to translocate to the cell membrane. After 1 h of AT_1R activation, although some perimembrane pits and intracellular vesicles formed (in 40% of cells, βarr1RRKK/Q remained at the cell surface, whereas in 60% of the cells, it trafficked to vesicles), the robust development of clustered, perinuclear deep-core vesicles was not as apparent compared with the wild-type βarr1. Thus, the hydrophobicity of helix I seems to contribute more than the charged residues to the membrane translocation of βarr1.

Figure 3B shows the trafficking of wild-type and mutant βarr2-GFP after AT_1R activation. Wild-type βarr2-GFP dis-

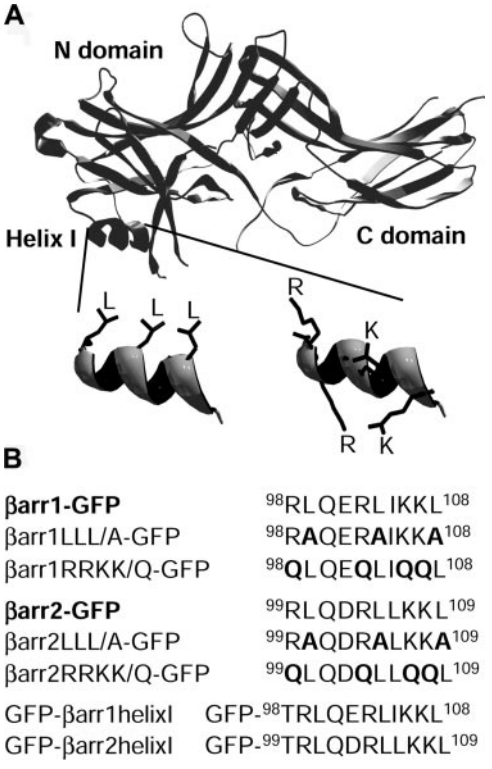

Fig. 1. Arrestin structure and various site-directed mutants at helix I. A, high-resolution X-ray structure of β-arrestin (1G4M) (Han et al., 2001) was generated using Swiss Pdb Viewer (http://www.expasy.ch/spdbv) and rendered using POV-Ray (http://www.povray.org). Helix I within the N domain of β-arrestin has hydrophobic residues (Leu100, Leu104, and Leu108, βarr1 numbering) aligned on one side and positive charges (Arg99, Arg103, Lys106, and Lys107) aligned on the opposite side. B, amino acid sequences of helix I of wild-type and mutant βarr1 and -2. In addition, the isolated helix I of both βarr1 and -2 was inserted at the C terminus of GFP.

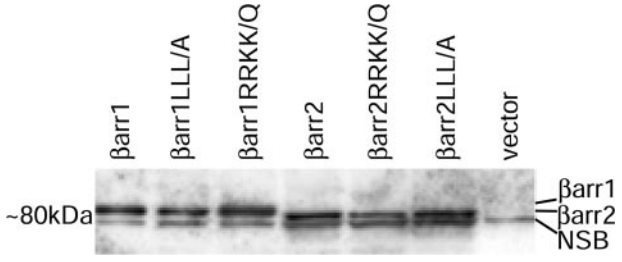

Fig. 2. Expression of wild-type and mutant β-arrestins. Samples of cell extracts expressing wild-type and mutant β-arrestins were probed with anti-GFP antibody to confirm equal expression. The position of β-arrestins are indicated, as is a nonspecific band (NSB).

played complete translocation to the membrane followed by subsequent robust redistribution to endocytic vesicles (observed in all 50 cells). In contrast, βarr2LLL/A-GFP translocated to the cell surface and formed pits at the membrane (60 min) but did not traffic strongly into cytoplasmic deep-core vesicles (20% vesicular, 80% membrane-localized). Likewise, the βarr2RRKK/Q mutant translocated to the cell periphery in response to AngII stimulation. After 1 h of receptor activation, most βarr2RRKK/Q persisted at the membrane, with only some small pits/vesicles observed (26% vesicular, 74% membrane-localized). Together, these results indicate that mutation of helix I mostly affects postmembrane sorting of β-arrestins after AngII stimulation. Both hydrophobic and positively charged facets of helix I seem to serve a vital role in postmembrane events for both β-arrestin isoforms.

To examine the effect of mutating helix I of β-arrestin on the trafficking pattern of the AT_1R, we coexpressed an AT_1R-EGFP receptor with either wild-type βarr2-HcRed or βarr2LLL/A-HcRed. As shown in Fig. 4, AngII stimulation promoted internalization of the AT_1R into deep-core endocytic vesicles where it is colocalized with the wild-type β-arrestin. When coexpressed with βarr2LLL/A-HcRed, the AT_1R-GFP moved from the cell surface to small pits/vesicles, which also contained βarr2LLL/A-HcRed; however, these did not resemble coalesced, deep-core vesicles.

To determine whether mutations in helix I can also affect arrestin trafficking in class A GPCRs (which preferentially traffic βarr2), we next compared wild-type and mutant βarr2-GFP translocation after activation of the β_2AR. As shown in Fig. 5, stimulation of β_2AR, using the agonist isoproterenol, leads to the membrane targeting of wild-type βarr2 and the formation of membrane-localized pits; deep-core vesicles did not develop. Compared with the wild type, the β-arrestin mutant containing substitutions within the hydrophobic residues, βarr2LLL/A, translocated weakly to the cell membrane and remained in membrane-proximal pits/vesicles. In contrast, the βarr2RRKK/Q mutant that has mutations within the positively charged facet rapidly and more completely redistributed to the cell surface and remained there for up to 60 min after stimulation. These data support our findings that alterations in helix I do not prevent the translocation of β-arrestins to the plasma membrane after receptor stimulation.

GFP Fusions of Helix I Are Not Localized to the Plasma Membrane. To further investigate whether helix I is potentially amphitropic, we engineered constructs in which the 11 amino acid helix I of βarr1 and -2 was fused at the C terminus of GFP to generate GFP-βarr1helixI and GFP-βarr2helixI. Previous studies have shown that amphitropic segments can act in isolation to target reporter proteins to the cell membrane. A key example of this is the positively charged motif within the signaling molecule K-ras,

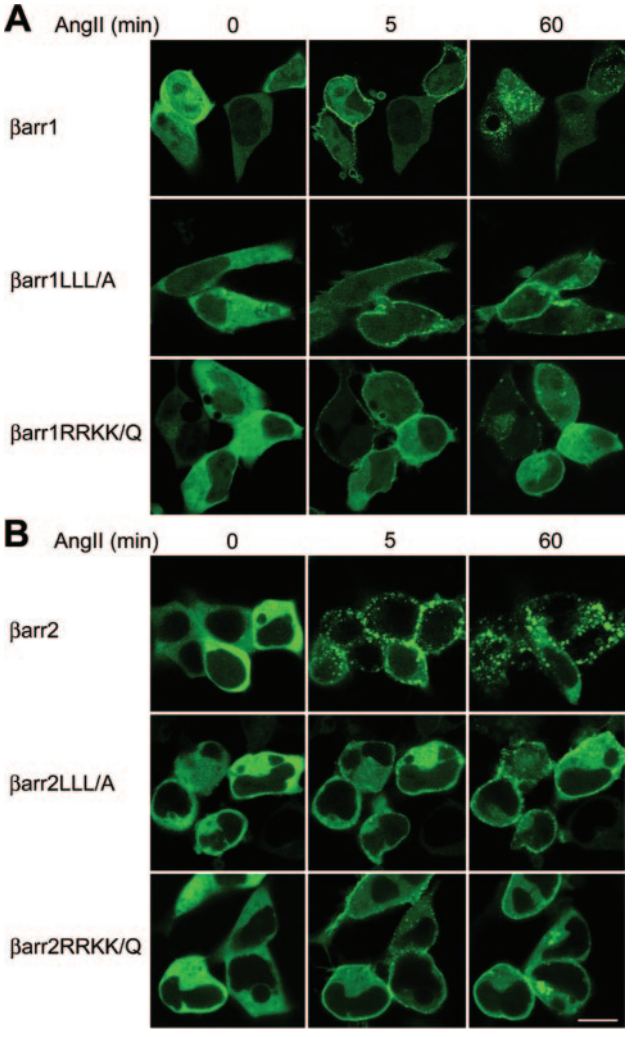

Fig. 3. Trafficking of β-arrestin helix I mutants upon AngII-induced stimulation by a class B GPCR. AT_1R was transiently coexpressed with either wild-type and mutant βarr1-GFP (A) or wild-type and mutant βarr2-GFP (B) in HEK-293 cells. Cells were stimulated with the agonist AngII (100 nM) at 37°C. The distribution of βarr-GFP and mutants was visualized under confocal microscopy before (0 min) and after stimulation up to 60 min. The results shown are representative of four experiments. Bar, 10 μm.

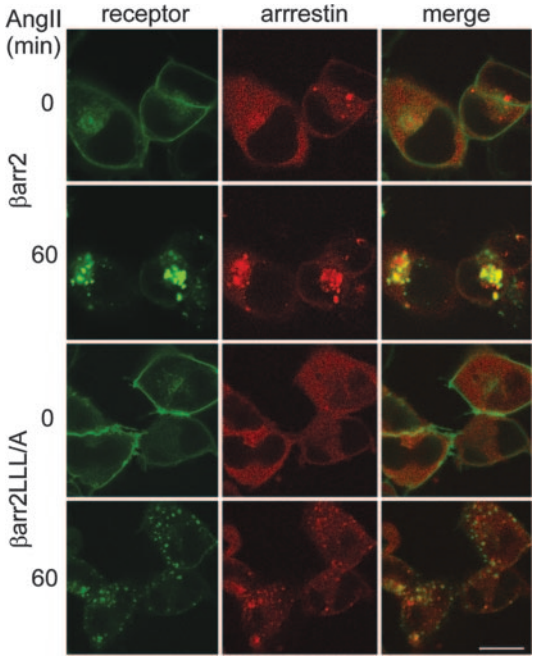

Fig. 4. Colocalization of the AT_1R with wild-type βarr2 or β-arr2LLL/A. The AT_1R-GFP was cotransfected with either wild-type βarr2-HcRed or βarr2LLL/A-HcRed in HEK-293 cells. Cells were stimulated with AngII (100 nM) at 37°C and viewed using confocal microscopy before (0 min) and after stimulation (60 min). The results shown are representative of four experiments. Bar, 10 μm.

which promotes strong plasma membrane localization when fused to GFP (tK-GFP) (Apolloni et al., 2000). We expressed the positive control, tK-GFP, and our GFP fusion constructs of helix I from βarr1 and βarr2 in HEK-293 cells and examined their cellular location using confocal microscopy (Fig. 6). In contrast to tK-GFP, which is confined exclusively to cell surface, both GFP-βarr1helixI and GFP-βarr2helixI showed a diffuse cytoplasmic localization. This indicates that helix I alone is not sufficient to target membrane anchoring.

Interaction of AT_1R with Helix I Mutants. Confocal microscopy can visually detect β-arrestin trafficking but does not provide information on the direct association of β-arrestin with receptors. Given the altered trafficking of mutant helix I β-arrestins in response to AT_1R activation, we wondered whether these mutants maintained the capacity to interact with activated AT_1R and affect function. Hence, we engineered the AT_1R to contain R. *reniformis* luciferase (AT_1R-Rluc) as a C-terminal fusion protein and measured protein-protein interactions with wild-type and mutant versions of βarr-GFP via BRET in living cells. Upon AngII stimulation, cells containing AT_1R and either wild-type βarr1 or βarr2 showed a similar increase in BRET signal (Fig. 7A). For comparison, BRET signaling for both βarr1 and βarr2 was confirmed using the TRHR1 as a positive control—this receptor is a class B receptor that has been reported previously to interact strongly with both βarr1 and βarr2 (Hanyaloglu et al., 2002). As shown in Fig. 7B, mutation in either the hydrophobic or positively-charged aspects of helix I does not severely impair the strength of the receptor-arrestin interaction measured after AngII stimulation.

We also used a long-acting luciferase substrate, EnduRen, to examine the kinetics and stability of the receptor-arrestin interactions over a 1-h time course. Immediately after AngII stimulation, βarr1 (Fig. 7C) and βarr2 (Fig. 7D), as well as their LLL/A and RRKK/Q mutants, rapidly associated with the AT_1R receptor. The association of wild-type β-arrestins was stable and maintained over 60 min, whereas the interaction with the mutants was less so.

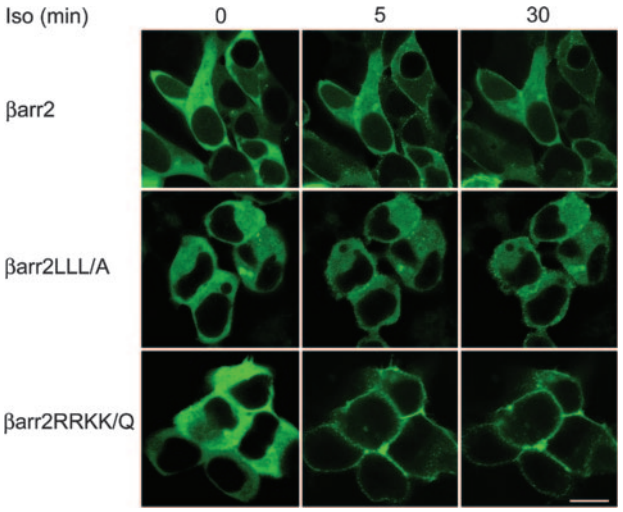

Fig. 5. Trafficking of βarr2 helix I mutants by a class A receptor. β_2AR and either wild-type or mutant βarr2-GFP were transfected into HEK-293 cells. Cells were stimulated with 10 μM of isoproterenol (Iso) at 37°C and viewed under confocal microscopy before (0 min) and after stimulation up to 30 min. The results shown are representative of four experiments. Bar, 10 μm.

Helix I Mutants Support AT_1R Internalization. Given that β-arrestin mutants translocate to the membrane but do not traffic effectively into deep-core vesicles, we investigated whether they could support AT_1R internalization or indeed hinder associations with endogenous arrestins, thereby interfering with receptor internalization. The latter possibility is supported by our observation that helix I mutants still complex with the AT_1R. As shown in Fig. 8, expression of wild-type and helix I mutants of both βarr1 (Fig. 8A) and βarr2 (Fig. 8B) in HEK-293 cells did not affect the rapid and robust internalization (~80% after 20-min stimulation) of the AT_1R. These data suggest that helix I mutants do not act in a dominant-negative manner. For comparison, in this assay, a well-established βarrestin dominant-negative ($\beta arr1^{319–418}$) that binds and sequesters clathrin causes a significant reduction of AT_1R internalization (Qian et al., 2001).

We next assessed whether these mutants could rescue receptor internalization in a situation in which endogenous β-arrestins were absent. For this we used an MEF cell line derived from βarr1 and βarr1/2KO mice (Kohout et al., 2001). It has been demonstrated previously that AT_1R internalization is abrogated in this cell line and that ectopic expression of wild-type βarr1 and βarr2 can rescue receptor endocytosis (Kohout et al., 2001). As expected, βarr1/2KO MEF lines transfected with AT_1R alone exhibited a dramatic reduction in receptor internalization (Fig. 9, A and B). Coexpression of either βarr1 or βarr2 restored receptor internalization, confirming β-arrestin–dependent endocytosis for this receptor. It is interesting that LLL/A and RRKK/Q mutants of both βarr1 and -2 supported receptor internalization in the βarr1/2KO line.

Discussion

The major outcome of the present study is that the amphipathic α-helix I of β-arrestin is not strongly amphitropic, contrary to previous conjecture (Han et al., 2001). The mutations in both hydrophobic and positively charged facets did not abrogate translocation to the membrane. Moreover, the isolated helix was unable to localize GFP to the plasma membrane, as could be demonstrated for another well-established amphitropic sequence (K-ras). The most obvious effect of mutating helix I was that β-arrestins trafficked poorly beyond their initial translocation to the membrane, and few deep-core endocytic vesicles were observed. This occurred despite the capacity of β-arrestin mutants to bind the AT_1R and support its rapid internalization. We conclude from this that functionally, with respect to trafficking, β-arrestins are only required at the membrane and that helix I is not strictly essential for receptor internalization. Whether these mutants are inhibited in respect to other β-arrestin functions

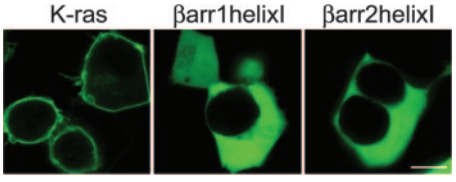

Fig. 6. GFP fusions of helix I are not localized to the plasma membrane. HEK-293 cells were transfected with either GFP-βarr1helixI, GFP-βarr2helixI, or membrane-bound K-ras-GFP (referred to as tK-GFP) as control and visualized using confocal microscopy. Bar, 10 μm.

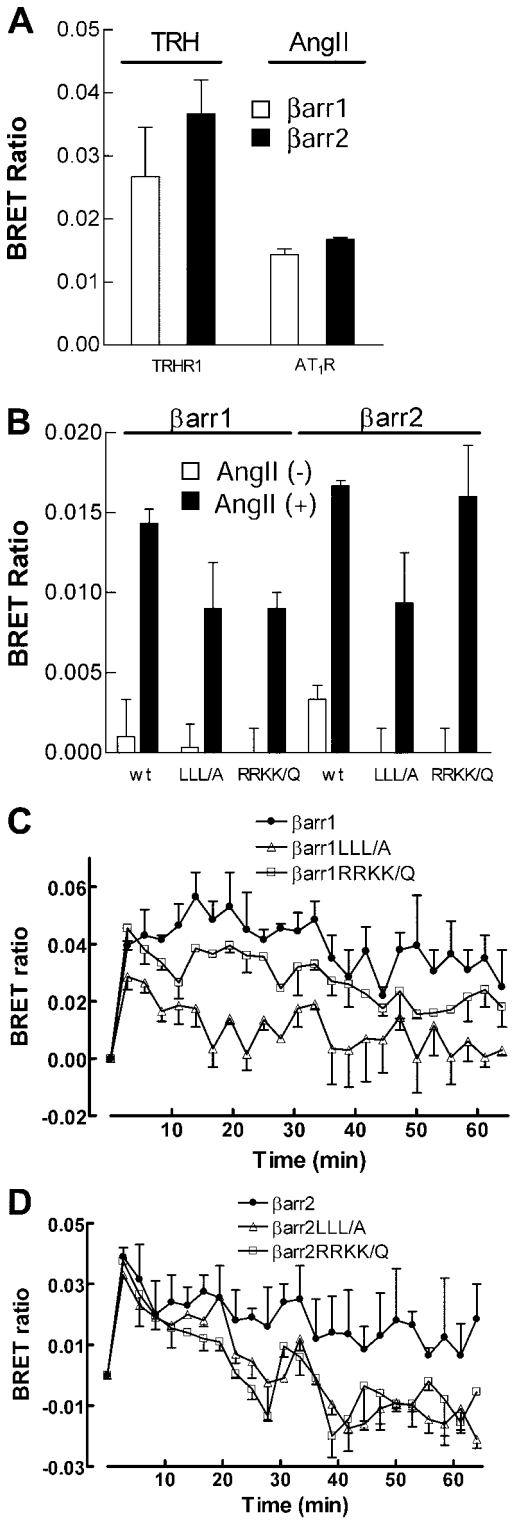

Fig. 7. Interaction of AT_1R with β-arrestin helix I mutants using BRET. COS-7 cells were cotransfected with TRHR1-Rluc or AT_1R-Rluc and either wild-type (wt) βarr1-GFP or βarr2-GFP (A) or were coexpressed with AT_1R-Rluc and either wild-type βarr-GFP or mutants (B). Cells were incubated with 5 μM coelenterazine, and BRET signals were measured between 0 and 10 min after thyrotropin-releasing hormone (1 μM) or AngII (100 nM) stimulation. To examine the kinetics of receptor-arrestin interaction, cells expressing AT_1R-Rluc and either wild-type βarr1-GFP (C) and βarr2-GFP (D), and their respective helix I mutants, were also incubated with 60 μM EnduRen (a long-acting luciferase substrate), and BRET signals were measured continuously for 60 min after AngII (100 nM) stimulation. Results shown are the mean ± S.E. of three separate experiments.

(e.g., scaffolding of signaling/regulatory molecules) (Luttrell et al., 1999; DeFea et al., 2000; Hall and Lefkowitz, 2002) has not been explored in this study but would be of interest.

Few studies have directly investigated the role of helix I in arrestin function. Structural data revealed that helix I of arrestin is normally constrained in a hydrophobic pocket within the N domain and that it is involved in holding arrestin in its basal state (Vishnivetskiy et al., 2000; Han et al., 2001; Gurevich and Gurevich, 2004). Han et al. (2001) predicted that helix I is displaced from the pocket after β-arrestin activation, allowing it to serve as an additional membrane anchor or to enhance receptor binding. However, this was not tested experimentally. Using purified rhodopsin in in vitro arrestin binding assays, Vishnivetskiy et al. (2000) reported that mutation of the leucines (to alanines) in helix I caused a modest increase in the constitutive activity (i.e., receptor binding) of arrestin. This constitutive activity was not enough for us to observe as an appreciable basal translocation of any of the β-arrestin mutants (either with substitutions in the hydrophobic or positively charged faces) because these mutants were found to distribute uniformly in the cytoplasm, like wild-type β-arrestins. In addition, these mutants did not display increased basal binding to receptor, in our hands, using BRET assays, which are a direct measure-

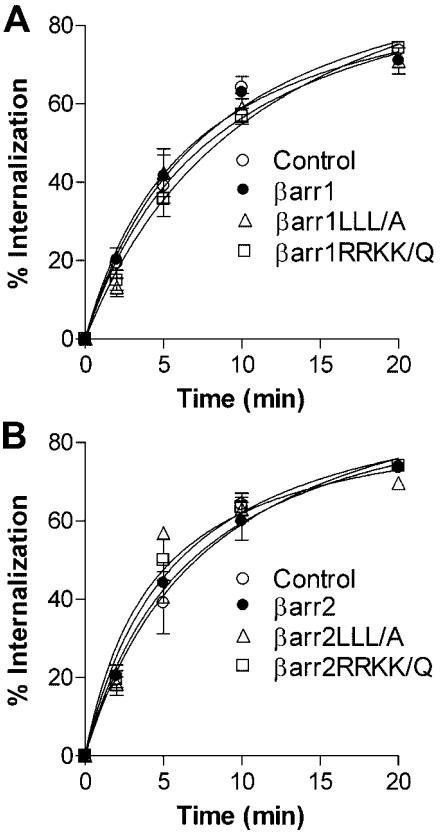

Fig. 8. β-arrestin helix I mutants do not act in a dominant-negative manner. HEK-293 cells were transiently transfected with AT_1R and either with or without wild-type or mutant βarr1 (A) or wild-type or mutant βarr2 (B). Cells were incubated with [^{125}I]AngII at 37°C for the indicated times. Bound ligand was stripped from the cell surface receptors, and the amount of intracellular radioactivity was expressed as a percentage of the total specific binding (intracellular + cell surface). Radioligand internalization was compared for wild-type and mutant β-arrestins in the presence of AT_1R. Data are mean ± S.E. of three experiments.

ment of receptor-arrestin interaction in living cells. After AngII stimulation, the arrestin mutants trafficked to the membrane. Furthermore, helix I in isolation (as a GFP fusion construct) was not strongly membrane-targeted (Fig. 6). It is clear that other parts of β-arrestin must be more important in this regard. It is of interest that residues within β-arrestin (K233Q, R237Q, and K251Q) have been shown to bind to phosphoinositides, and β-arrestin mutants lacking the phosphoinositides binding sequence do not traffic into pits or support receptor internalization (Gaidarov et al., 1999) and prevent arrestin trafficking and light adaptation in *Drosophila melanogaster* (Lee et al., 2003). A similar phenotype is observed with mutants lacking a binding site (R394A and R396A) for the clathrin-adaptor molecule (Laporte et al., 2000). Thus, β-arrestin binding to phosphoinositides or clathrin-adaptor molecule is an essential initial step in the endocytic pathway.

Rather than membrane targeting, the function of helix I in β-arrestin activity seemed to correlate more closely with postendocytic routing. Unlike the wild-type proteins, mutant β-arrestins were retained near the cell surface, presumably because helix I contributes to the processes that target arrestins into deep-core endocytic vesicles. Despite this increased accumulation at the membrane, expression of these mutant arrestins did not alter AT_1R binding and internalization. All helix I mutants rapidly interacted with activated AT_1Rs, as measured in BRET assays, and helix I mutants did not compete with endogenous β-arrestins for receptor binding in HEK-293 cells. Thus, they do not behave in a dominant-negative manner. Moreover, these mutants all fully supported AT_1R internalization in experiments using MEF cell lines deficient in both βarr1 and -2. Consistent with other reports (Kohout et al., 2001), AT_1R internalization was impaired in βarr1/2KO MEF cells, whereas re-expression of either wild-type βarr1 or -2 fully rescued receptor internalization, confirming β-arrestin–dependent endocytosis for this receptor. Our data clearly demonstrate that, despite their modified trafficking, β-arrestin mutants retained the capacity to efficiently drive AT_1R internalization. Thus, the integrity of helix I is not paramount for arrestin binding to the receptor or for the promotion of receptor internalization. Instead, helix I was shown to be critical for normal trafficking. This may reflect a role for helix I in the long-term stability of receptor-arrestin complexes and trafficking to endocytic vesicles.

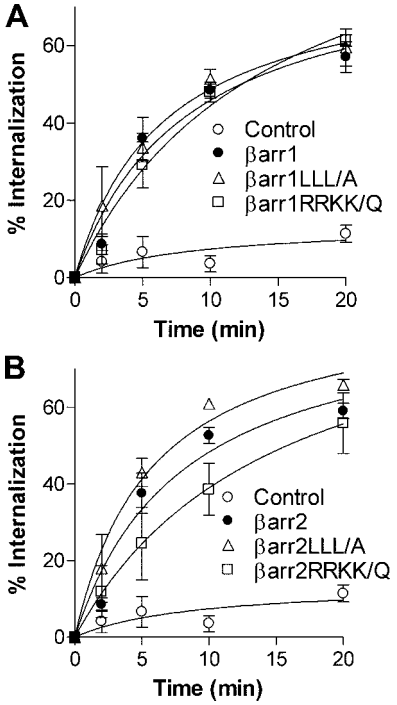

Fig. 9. β-arrestin helix I mutants support AT_1R internalization. AT_1R were coexpressed with or without either wild-type or mutant βarr1 (A) or wild-type or mutant βarr2 (B) in βarr1/2 KO MEF cell lines, and cells were incubated with [^{125}I]AngII at 37°C for specified times. Bound ligand was stripped from the cell surface receptors, and the amount of intracellular radioactivity was expressed as a percentage of the total specific binding (intracellular + cell surface). Radioligand internalization was compared for wild-type and mutant β-arrestins in the presence of AT_1R. Data are mean ± S.E. of three experiments.

We were surprised that helix I was not amphitropic, especially considering recent evidence that many proteins involved in GPCR signaling contain amphitropic sequences that allow reversible recruitment to the receptor signaling complex. For example, RGS4 and RGS16 proteins, which are involved in enhancing the GTPase catalytic activity of G proteins, require an amphipathic α-helix for plasma membrane association (Chen et al., 1999; Bernstein et al., 2000). Likewise, Thiyagarajan et al. (2004) demonstrated that GRK5, which interacts and phosphorylates GPCRs, also contains an amphipathic α-helix, which helps to tether GRK5 to the cytoplasmic membrane. This helix in isolation (fused to GFP) mediated membrane localization, and mutations at this helix disrupted membrane targeting (Thiyagarajan et al., 2004). Moreover, ADP-ribosylation factor, a vesicular trafficking regulator, binds to the lipid bilayer with high affinity via a basic amphipathic α-helix (Johnson and Cornell, 1999). On the other hand, alterations of the membrane binding motif eliminated membrane interaction (Antonny et al., 1997). Finally, we and others have observed a key role for helix VIII (a positively charged amphipathic helix) in the proximal carboxyl terminus of GPCRs in membrane tethering and receptor activation (Krishna et al., 2002; Mozsolits et al., 2002; Zhong et al., 2004).

In summary, helix I of β-arrestin is not strongly amphitropic. In contrast, mutants bearing changes in this helix still traffic to the cell surface, although they seem to be blocked in their capacity to strongly target into deep-core vesicles. Given that these mutants fully support AT_1R internalization, we predict that the major function of helix I is postmembrane endocytic targeting rather than amphitropism.

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