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Helix I of β-arrestin is involved in post-endocytic trafficking, but is not required for membrane translocation, receptor binding and internalization

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Abbreviations: GPCRs, G protein-coupled receptors; AngII, angiotensin II; AT_1R, type 1 AngII receptor; GFP, green fluorescent protein; β_2AR, β_2-adrenergic receptor; RGS, Regulators of G protein Signaling; GRKs, GPCR kinases; Rluc, Renilla Luciferase; TRH, thyrotropin-releasing hormone; βarr, β-arrestin; MEFs, mouse embryonic fibroblasts; BRET, bioluminescence resonance energy transfer; AP2, clathrin-adapter molecule; Iso, isoproterenol.

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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$_1$R), to promote receptor desensitization and internalization. The AT$_1$R is a *class B* GPCR that recruits both β-arrestin1 and β-arrestin2, forming stable complexes that co-traffic 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 have 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. Following 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 to 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. BRET analysis revealed that both wild type and β-arrestin mutants retained a capacity to interact with the AT$_1$R, 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 post-membrane trafficking, but is not strongly amphitropic.

INTRODUCTION

The non-visual 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 et al., 2002). The recruitment and trafficking of β-arrestins can be visualized using confocal microscopy of green fluorescent protein (GFP)-labeled βarr1 and 2. Based on preferential trafficking of βarr1 and 2, Oakley *et al*. (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 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 (Vishnivetskiy et al., 2004; Gurevich and Gurevich, 2004). Although the structural changes that underpin arrestin activation are poorly understood, it is predicted that this process likely 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 thought 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 (L^{100}, L^{104}, L^{108}, βarr1 numbering) aligned on one side of the helix and positive charges (R^{99}, R^{103}, K^{106}, K^{107}) 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 appear 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 thought 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 have investigated the role of helix I in β-arrestin trafficking, receptor interaction and function. We observe that mutations to helix I that negate the hydrophobic and charged aspects do not abrogate membrane recruitment of arrestins in response to receptor

activation. Interestingly, 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.

EXPERIMENTAL PROCEDURES

Materials

Anti-GFP polyclonal antibody was purchased from Clontech Laboratories, Inc. and AngII was from Auspep Pty. Ltd., Melbourne, Australia. The SuperSignal West Pico Chemiluminescent was purchased from Pierce. Radiolabeled [^{125}I]-AngII (specific activity > 2000 Ci/mmol) was provided by ProSearch, Melbourne, Australia. All other chemicals were from Sigma or BDH Laboratory Supplies.

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_2-adrenergic receptor (β_2AR) plasmid was provided by R. Summers, Department of Pharmacology, Monash University, Melbourne, Australia. The construction of hemagglutinin epitope (HA)-tagged wild type $AT_{1A}R$, an AT_1R-EGFP and the dominant-negative $\beta arr1$ ($\beta arr1^{318-419}$) have been reported previously (Thomas et al., 1998; Qian et al., 2001; Holloway et al., 2002). A thyrotropin-releasing hormone receptor (TRHR1)-Renilla Luciferase (Rluc) construct was described previously (Hanyaloglu et al., 2002) and the AT_1R-Rluc construct was generated by amplifying the HA-AT_1R using PCR (sense primer, T7 primer; antisense primer, 5'-GAA*GCGGCCG*CTCCACCTCAAAACAAGACGCAGG with an extra *Not*I site (bold 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) from $\beta arr1$-GFP, $\beta arr2$-GFP and $\beta arr2$-HcRed (kindly provided by M.G. Caron, Duke University Medical Center, Durham, NC). LLL/A mutant versions of $\beta arr1$-GFP and $\beta arr2$-GFP contained three substitutions of leucine residues with alanine ($\beta arr1$ L^{99}, L^{103}, L^{107}; $\beta arr2$ L^{100}, L^{104}, L^{108}), whereas RRKK/Q mutant versions of

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βarr1-GFP and βarr2-GFP are characterized by quadruple substitutions of arginine and lysine residues with glutamine (βarr1 R[98], R[102], K[105], K[106]; βarr2 R[99], R[103], K[106], K[107]). 5'-oligonucleotides used for mutagenesis were (5'-3'):

βarr1LLL/A   *CGG*GCCATCAAGAAGGCGGGCGAGCATGCCTACCCC (sense)

*CTC*TTGTGCCCGAGTCAGTGGCTTCTTGTC (antisense)

βarr2LLL/A   *GCC*CTGAAGAAGGCGGGCCAGCATGCCCACCCC (sense)

*CCG*GTCCTGTGCGCGGGTGGGGGGCCGAGG (antisense)

βarr1RRKK/Q   *CTG*ATCCAGCAGCTGGGCGAGCATGCCTACCCC (sense)

*CTG*CTCTTGTAGCTGAGTCAGTGGCTTCTTGTC (antisense)

βarr2RRKK/Q   *CTG*CTGCAGCAGTTGGGCCAGCATGCCCACCCC (sense)

*CTG*GTCCTGTAGCTGGGTGGGGGGCCGAGGTGG (antisense)

Oligonucleotides were 5'-phosphorylated using T4 polynucleotide kinase. Silent restriction sites (*BsrB*I, *Dra*II or *Pvu*II) were introduced to facilitate the screening of mutated clones (bold 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 *Hind*III/*Bam*HI cDNA fragments of βarr1 and 2, corresponding to helix I, into the cloning site of pEGFP-C1 (Clontech Laboratories, Inc.). 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) cells were maintained in DMEM 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 60-80 % confluence on 12-well culture plates. Cells were transiently co-transfected with 0.3 μg DNA/well of 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 (Life Technologies). For confocal experiments, cells were plated on collagen-coated 35 mm glass bottom dishes (MatTek Corporation, Ashland, MA), and transiently co-transfected with 30 ng DNA of either wild type βarr1-GFP, βarr2-GFP, βarr2-HcRed or mutants with either 70 ng 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 (Life Technologies). Cells were assayed 24 - 48 hours post-transfection.

Confocal microscopy

HEK-293 cells were changed into serum-free DMEM at least 2 hours before commencing confocal experiments. Localization and trafficking of βarrestins in response to AngII (100 nM) or isoproterenol (10 μM) stimulation was viewed with a 63 x 1.2 NA water immersion objective on a heated stage at 37°C. Images were collected using a Zeiss LSM 510 META confocal microscope. To compare trafficking between wild type and mutant β-arrestins following 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 these values as a percentage of the total number of cells examined.

Bioluminescence resonance energy transfer (BRET) assay

β-arrestin-AT_1R interaction in living cells was examined using BRET, as described previously (Kroeger et al., 2001; Hanyaloglu et al., 2002). Briefly, AT_1R-Rluc and either wild type βarr1-GFP, βarr2-GFP or mutants were co-expressed 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 renilla luciferase, coelenterazine (Molecular Probes). In addition, a time course study was performed in which cells were incubated in the presence of a long-acting substrate, EnduRen (Promega). Subsequently, cells were stimulated with AngII and consecutive readings were collected for 60 min. The binding of βarr1-GFP and βarr2-GFP to the AT$_1$R-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 nm and 515 nm. An increased ratio indicates β-arrestin-AT$_1$R interaction.

AT$_1$R internalization

Receptor internalization assays were determined as acid-insensitive [^{125}I]-AngII receptor binding as previously described (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) where hydrophobic residues on one face of the helix were substituted to alanine (βarr1LLL/A and βarr2LLL/A) and where 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), following 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. Interestingly, 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 hour 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

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compared to the wild type βarr1. Thus, the hydrophobicity of helix I seems to contribute more than the charged residues to the membrane translocation of βarr1.

Fig. 3B shows the trafficking of wild type and mutant βarr2-GFP following AT$_1$R activation. Wild type βarr2-GFP displayed 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). Similarly, the βarr2RRKK/Q mutant translocated to the cell periphery in response to AngII stimulation. After 1 hour 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 post-membrane sorting of β-arrestins after AngII stimulation. Both hydrophobic and positively-charged facets of helix I appear to serve a vital role in post-membrane events for both β-arrestin isoforms.

To examine the effect of mutating helix I of β-arrestin on the trafficking pattern of the AT$_1$R, we co-expressed an AT$_1$R-EGFP receptor with either wild type βarr2-HcRed or βarr2LLL/A-HcRed. As shown in Fig. 4, AngII stimulation promoted internalization of the AT$_1$R into deep-core endocytic vesicles, where is co-localized with the wild type β-arrestin. When co-expressed with βarr2LLL/A-HcRed, the AT$_1$R-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 following activation of the β$_2$-adrenergic receptor (β$_2$AR). As shown in Fig. 5, stimulation of β$_2$AR, 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 to 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 up to 60 minutes post-stimulation. These data support our findings that
alterations in helix I do not prevent the translocation of β-arrestins to the plasma membrane
following 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 where 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 signalling molecule K-ras, 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$_1$R 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 renilla 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 previously reported 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 utilized a long-acting, luciferase substrate, EnduRen, to examine the kinetics and

stability of the receptor-arrestin interactions over a 1 h time course. Immediately following

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.

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

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mutants do not act in a dominant/negative manner. For comparison, in this assay, a well-established βarrestin dominant/negative (βarr1[319-418]) that binds and sequesters clathrin causes a significant reduction of AT_1R internalization (Qian et al., 2001).

We next assessed if these mutants could rescue receptor internalization in a situation where endogenous β-arrestins were absent. For this we used a MEF cell line derived from βarr1 and βarr2 knockout (βarr1/2KO) mice (Kohout et al., 2001). It has previously been demonstrated 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. 9A and B). Co-expression of either βarr1 or βarr2 restored receptor internalization, confirming β-arrestin-dependent endocytosis for this receptor. Interestingly, 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 (eg. scaffolding of signaling/regulatory molecules) (Luttrell et al., 1999; DeFea et al., 2000; Hall et al., 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 (Han et al., 2001; Vishnivetskiy et al., 2000; Gurevich and Gurevich, 2004). Han *et al*. (Han et al., 2001) predicted that helix I is displaced from the pocket following 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*. (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) as

these mutants were found to distribute uniformly in the cytoplasm, like wild type β-arrestins.

Also these mutants did not display increased basal binding to receptor, in our hands, using BRET

assays, which are a direct measurement of receptor-arrestin interaction in living cells. Following

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). Clearly, other

parts of β-arrestin must be more important in this regard. It is of interest that residues within

β-arrestin ($K^{233}Q$, $R^{237}Q$, $K^{251}Q$) have been shown to bind to phosphoinositides, and β-arrestin

mutants lacking the phosphoinositides binding sequence do not traffic into pits nor support

receptor internalization (Gaidarov et al., 1999) and prevent arrestin trafficking and light

adaptation in *Drosophila* (Lee et al., 2003). A similar phenotype is observed with mutants

lacking a binding site ($R^{394}A$ and $R^{396}A$) for the clathrin-adaptor molecule (AP2) (Laporte et al.,

2000). Thus, β-arrestin binding to phosphoinositides or AP2 is an essential initial step in the

endocytic pathway.

Rather than membrane targeting, the function of helix I in β-arrestin activity appeared to

correlate more closely with post-endocytic 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 whilst 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$_1$R internalization. Thus, the integrity of helix I is not paramount for arrestin binding to the receptor, nor 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.

We were surprized 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). Similarly, Thiyagarajan *et al*. (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). Conversely, 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$_1$R

internalization, we predict that the major function of helix I is post-membrane endocytic

targeting rather than amphitropism.

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FIGURE LEGENDS

Figure 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 (hptt://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 (L^{100}, L^{104}, L^{108}, βarr1 numbering) aligned on one side and positive charges (R^{99}, R^{103}, K^{106}, K^{107}) 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.

Figure 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 non-specific band (NSB).

Figure 3. Trafficking of β-arrestin helix I mutants upon AngII-induced stimulation by a *Class B* GPCR. AT_1R was transiently co-expressed 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.

Figure 4. Colocalization of the AT_1R with wild type βarr2 or β-arr2LLL/A. The AT_1R-GFP was co-transfected 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.

Figure 5. Trafficking of βarr2 helix I mutants by a *Class A* receptor. β_2-adrenergic 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.

Figure 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.

Figure 7. Interaction of AT$_1$R with β-arrestin helix I mutants using BRET. COS-7 cells were co-transfected with TRHR1-Rluc or AT$_1$R-Rluc and either wild type (wt) βarr1-GFP or βarr2-GFP (A), or co-expressed with AT$_1$R-Rluc and either wild type βarr-GFP or mutants (B). Cells were incubated with 5 μM coelenterazine and BRET signals were measured between 0-10 min after thyrotropin releasing hormone (1 μM of TRH) or AngII (100 nM) stimulation. To examine the kinetics of receptor-arrestin interaction, cells expressing AT$_1$R-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.

Figure 8. β-arrestin helix I mutants do not act in a dominant negative manner. HEK-293 cells were transiently transfected with AT$_1$R and either with or without wild type (wt) or mutant βarr1 (A), wild type or mutant βarr2 (B). Cells were incubated with [^{125}I]AngII at 37°C for 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$_1$R. Data are mean ± S.E. of three experiments.

Figure 9. β-arrestin helix I mutants support AT$_1$R internalization. AT$_1$R were co-expressed with or without either wild type (wt) 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$_1$R. Data are mean ± S.E. of three experiments.

A

N domain

C domain

Helix I

L L L

R K

R K

B
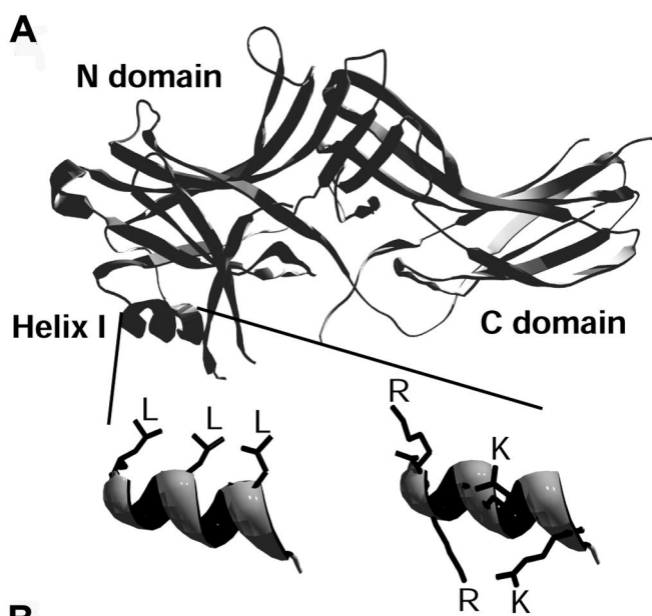
βarr1-GFP	[98]RLQERLIKKL[108]
βarr1LLL/A-GFP	[98]R**A**QER**A**IKK**A**[108]
βarr1RRKK/Q-GFP	[98]**Q**LQE**Q**LI**QQ**L[108]
βarr2-GFP	[99]RLQDRLLKKL[109]
βarr2LLL/A-GFP	[99]R**A**QDR**A**LKK**A**[109]
βarr2RRKK/Q-GFP	[99]**Q**LQD**Q**LL**QQ**L[109]
GFP-βarr1helixI	GFP-[98]TRLQERLIKKL[108]
GFP-βarr2helixI	GFP-[99]TRLQDRLLKKL[109]

	βarr1	βarr1LLL/A	βarr1RRKK/Q	βarr2	βarr2RRKK/Q	βarr2LLL/A	vector	
~80kDa								βarr1 βarr2 NSB

A   AngII (min)   0   5   60
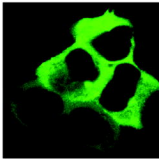
βarr1

βarr1LLL/A

βarr1RRKK/Q

B   AngII (min)   0   5   60

βarr2

βarr2LLL/A

βarr2RRKK/Q

Iso (min) 0 5 30

βarr2

βarr2LLL/A

βarr2RRKK/Q

| K-ras | βarr1helixI | βarr2helixI |

C

βarr1
βarr1LLL/A
βarr1RRKK/Q

D

βarr2
βarr2LLL/A
βarr2RRKK/Q

A

% Internalization (y-axis, 0–80)
Time (min) (x-axis, 0–20)

○ Control
● βarr1
△ βarr1LLL/A
□ βarr1RRKK/Q

B

% Internalization (y-axis, 0–80)
Time (min) (x-axis, 0–20)

○ Control
● βarr2
△ βarr2LLL/A
□ βarr2RRKK/Q

A

% Internalization vs Time (min)

- ○ Control
- ● βarr1
- △ βarr1LLL/A
- □ βarr1RRKK/Q

B

% Internalization vs Time (min)

- ○ Control
- ● βarr2
- △ βarr2LLL/A
- □ βarr2RRKK/Q