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Cellular Trafficking of G Protein-coupled Receptor/β-Arrestin Endocytic Complexes*

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Jie Zhang‡, Larry S. Barak‡, Pieter H. Anborgh§¶, Stephane A. Laporte‡‖, Marc G. Caron‡, and Stephen S. G. Ferguson§ ‡‡**

From the ‡Howard Hughes Medical Institute Laboratories, Departments of Cell Biology and Medicine, Duke University Medical Center, Durham, North Carolina 27710 and the §John P. Robarts Research Institute, Departments of Physiology and Pharmacology and Toxicology, University of Western Ontario, P.O. Box 5015, 100 Perth Drive, London, Ontario N6A 5K8, Canada

β-Arrestins are multifunctional proteins identified on the basis of their ability to bind and uncouple G protein-coupled receptors (GPCR) from heterotrimeric G proteins. In addition, β-arrestins play a central role in mediating GPCR endocytosis, a key regulatory step in receptor resensitization. In this study, we visualize the intracellular trafficking of β-arrestin2 in response to activation of several distinct GPCRs including the β_2-adrenergic receptor (β_2AR), angiotensin II type 1A receptor ($AT_{1A}R$), dopamine D1A receptor ($D_{1A}R$), endothelin type A receptor (ET_AR), and neurotensin receptor (NTR). Our results reveal that in response to β_2AR activation, β-arrestin2 translocation to the plasma membrane shares the same pharmacological profile as described for receptor activation and sequestration, consistent with a role for β-arrestin as the agonist-driven switch initiating receptor endocytosis. Whereas redistributed β-arrestins are confined to the periphery of cells and do not traffic along with activated β_2AR, $D_{1A}R$, and ET_AR in endocytic vesicles, activation of $AT_{1A}R$ and NTR triggers a clear time-dependent redistribution of β-arrestins to intracellular vesicular compartments where they colocalize with internalized receptors. Activation of a chimeric $AT_{1A}R$ with the β_2AR carboxyl-terminal tail results in a β-arrestin membrane localization pattern similar to that observed in response to β_2AR activation. In contrast, the corresponding chimeric β_2AR with the $AT_{1A}R$ carboxyl-terminal tail gains the ability to translocate β-arrestin to intracellular vesicles. These results demonstrate that the cellular trafficking of β-arrestin proteins is differentially regulated by the activation of distinct GPCRs. Furthermore, they suggest that the carboxyl-tail of the receptors might be involved in determining the stability of receptor/β-arrestin complexes and cellular distribution of β-arrestins.

Signal transduction via G protein-coupled receptors (GPCRs)[1] is intimately associated with a wide variety of biological processes including neurotransmission, chemoattraction, cardiac function, olfaction, and vision. β-Arrestin proteins play an important role in regulating the responsiveness of GPCRs by contributing to mechanisms involved in both GPCR desensitization and resensitization (1–5). β-Arrestins regulate GPCR desensitization by binding and uncoupling the receptors from heterotrimeric G proteins once they have been phosphorylated by G protein-coupled receptor kinases (GRKs) (1, 3). In addition, they are also required for the sequestration (endocytosis) of a growing number of GPCRs, including the CCR-5, follitropin receptor, lutropin/choriogonadotropin receptor, m2 muscarinic acetylcholine receptor, mu opioid receptor, substance P receptor, and the β_2-adrenergic receptor (β_2AR) (4, 6–11). At least in the case of the β_2AR, the agonist-dependent sequestration of the receptor to an endosomal compartment not only promotes receptor dephosphorylation but is essential for the re-establishment of normal receptor responsiveness (5, 12–15).

Recent studies suggest that β-arrestins participate in GPCR sequestration by directing receptors to clathrin-coated vesicles (4, 16, 17). β-Arrestins have been shown to undergo redistribution in response to receptor activation both in live cells and following the fixation of cells, and to co-localize with clathrin (7, 11, 18, 19). However, while the phenomenon of β-arrestin cellular trafficking is potentially important for understanding mechanisms underlying GPCR internalization and resensitization, the detailed pharmacology of the receptor-mediated β-arrestin redistribution has never been characterized. As a consequence, it is not clear whether the pharmacological profile of β-arrestin translocation recapitulates the pharmacology described for GPCR endocytosis. Moreover, the cellular fate of β-arrestin proteins following association with various GPCRs also remains unknown, as well as where and when β-arrestins dissociate from each receptor. In the case of rhodopsin, it was demonstrated that the interaction of visual arrestin with rhodopsin prevented the dephosphorylation and resensitization of the receptor (20, 21). Therefore, it is likely that the dissociation

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** Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute, Box 3287, Duke University Medical Center, Durham NC 27710. Tel.: 919-684-5433; Fax: 919-681-8641.

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; $AT_{1A}R$, angiotensin II type 1A receptor; $AT_{1A}R$-β_2AR-CT, chimeric $AT_{1A}R$ mutant with the β_2AR carboxyl-terminal tail; β_2AR, β_2-adrenergic receptor; β_2AR-$AT_{1A}R$-CT, chimeric β_2AR mutant with the $AT_{1A}R$ carboxyl-terminal tail; βarr$_2$GFP, green fluorescent protein conjugate of β-arrestin2; βarr$_1$GFP, green fluorescent protein conjugate of β-arrestin1; $D_{1A}R$, dopamine D1A receptor; ET_AR, endothelin type A receptor; GFP, green fluorescent protein; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; MEM, minimal essential medium; NTR, neurotensin receptor.

of the β-arrestin/receptor complex contributes to the regulation of responsiveness for other GPCRs.

In the present study, we used a green fluorescent protein conjugate of β-arrestin2 (βarr_2GFP) (18) to examine the cellular trafficking of β-arrestin upon stimulation of several distinct GPCRs. Our data demonstrate that the pharmacology of β-arrestin2 translocation in living cells could account for the agonist dependence of β_2AR sequestration. Moreover, β-arrestin2 was observed to redistribute to distinct subcellular locations in response to activation of different GPCRs. This differential redistribution of β-arrestins likely involves the function of the carboxyl-terminal region of the receptors.

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney (HEK 293) cells were provided by the American Type Culture Collection (ATCC). Tissue culture media and fetal bovine serum were obtained from Life Technologies, Inc. Isoproterenol and dopamine were purchased from Research Biochemicals International. Endothelin and neurotensin were from Peninsula Laboratories, and angiotensin II was from Sigma. Rabbit anti-HA polyclonal antibody and mouse anti-HA 12CA5 monoclonal antibody were obtained from Babco and Boehringer Mannheim, respectively. Fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody was purchased from Sigma, and rhodamine-conjugated goat anti-rabbit Fabs was obtained commercially from Organon Teknika. [^{125}I]Cyanopindolol was purchased from NEN Life Science Products.

DNA Construction—All recombinant DNA procedures were carried out following standard protocols. β-Arrestin1 with GFP conjugated to its COOH terminus (βarr_1GFP) was constructed in a manner similar to βarr_2GFP (18) by replacing the terminal stop codon of β-arrestin1 with a *Sal*I restriction site and inserting the modified cDNA in frame into the polylinker of p(S65T)GFP-N3 (CLONTECH) (18). pcDNA3-AT_{1A}R-β_2AR-CT and pcDNA1-Amp-β_2AR-AT_{1A}R-CT were constructed by polymerase chain reaction. The chimeric $AT_{1A}R$ with the β_2AR carboxyl-terminal tail ($AT_{1A}R$-β_2AR-CT) contains the first 302 amino acids (Met^1–Tyr^{302}) of the $AT_{1A}R$ fused to the last 87 amino acids (Cys^{327}–Leu^{413}) of the β_2AR. The chimeric β_2AR with the $AT_{1A}R$ carboxyl-terminal tail (β_2AR-AT_{1A}R-CT) includes the first 348 amino acids (Met^1–Lys^{348}) of the β_2AR fused to the last 38 amino acids (Ala^{324}–Glu^{359}) of the $AT_{1A}R$. The sequences of the DNA constructs were confirmed by DNA sequencing.

Cell Culture and Transfection—HEK 293 cells were grown in Eagle's minimal essential medium with Earle's salt (MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and gentamicin (100 μg/ml). The cells were seeded at a density of 2.5×10^6 cells/100-mm dish and transiently transfected with the cDNAs described in the figure legends by a modified calcium phosphate method (22). Following transfection (~18 h), the cells were incubated with fresh medium and allowed to recover 7–9 h before being reseeded into 35-mm glass-bottomed culture dishes (MatTek) or into six-well dishes (Falcon) containing or not containing 22-mm square glass coverslips coated with collagen (Sigma). The use of HEK 293 cells expressing β_2AR-GFP was described previously (23).

Receptor Expression—β_2AR expression was measured by saturating [^{125}I]cyanopindolol binding done at 30 °C for 60 min (24). The expression of other receptors was measured by flow cytometry and normalized according to the β_2AR expression measured at the same time by both flow cytometry and saturating binding (16). The receptor expression levels was between 2000 and 4000 fmol/mg of whole cell protein for the experiments assessing β-arrestin translocation with confocal microscope (see below) and between 1000 and 2000 fmol/mg of whole cell protein for all other experiments.

Confocal Microscopy—Confocal microscopy was performed on a Zeiss LSM-410 laser scanning microscope using either Zeiss 40 × 1.3 or Zeiss 63 × 1.4 numerical aperture oil immersion lenses. For characterizing the pharmacology of β-arrestin translocation in living cells, HEK 293 cells expressing β_2AR and low levels of βarr_2GFP were plated on 35-mm glass-bottomed culture dishes and kept warm at 30 °C in serum-free MEM on a heated microscope stage. βarr_2GFP fluorescent signals were collected sequentially using the Zeiss LSM software time scan function in the photon counting mode using single line excitation (488 nm). Drugs were applied to the cells either prior to or during the scanning of βarr_2GFP labeled cells. For studying β-arrestin trafficking in response to activation of other receptors, HEK 293 cells expressing the receptor of interest and low levels of βarr_2GFP were seeded on 22-mm square glass coverslips, stimulated with saturating concentrations of drugs at 30 °C for 1 h or as indicated, and then fixed with 3.7% paraformaldehyde in phosphate-buffered saline. βarr_2GFP fluorescent signals were collected using single line excitation (488 nm). Colocalization studies of βarr_2GFP and rhodamine-labeled receptor fluorescence were performed using dual excitation (488, 568 nm) and emission (515–540 nm, GFP; 590–610 nm, rhodamine) filter sets. Specificity of labeling and absence of signal cross-over were established by examination of single-labeled samples.

Immunofluorescent Labeling—For performing colocalization studies of βarr_2GFP and rhodamine-labeled β_2AR in live cells, HEK 293 cells expressing HA epitope-tagged β_2AR and βarr_2GFP grown on 35-mm glass-bottomed culture dishes were incubated in serum-free MEM containing anti-HA polyclonal antibody at 37 °C for 30 min. Cells were washed three times with ice-cold MEM and incubated for 30 min on ice in the presence of rhodamine-conjugated goat anti-rabbit Fabs. Cells were washed an additional three times with serum-free MEM at 30 °C and imaged by confocal microscopy as described above. For studying colocalization of βarr_2GFP and $AT_{1A}R$, HEK 293 cells expressing HA epitope-tagged $AT_{1A}R$ were grown on 22-mm square glass coverslips and incubated in serum-free MEM containing rhodamine-conjugated anti-HA 12CA5 monoclonal antibody on ice for 45 min. Cells were then washed, stimulated with a saturating concentration (500 nM) of angiotensin II for 1 h, washed again, fixed with 3.7% paraformaldehyde in phosphate-buffered saline, and imaged by confocal microscopy as described above.

Data Analysis—The changes in βarr_2GFP distribution to the plasma membrane were analyzed with IP Labs software. The magnitude of βarr_2GFP fluorescence at the plasma membrane were determined by integration of the fluorescence signal along the cell perimeter. The relative magnitude of βarr_2GFP distribution along a linear slice of the cell was quantitated by the line scan function provided with the Zeiss LSM 410 image analysis software. βarr_2GFP translocation time course and dose-response curves were analyzed using GraphPad Prism. All data points represent the mean ± S.D.

RESULTS

As described previously (18), in the absence of receptor activation βarr_2GFP fluorescence was evenly distributed throughout the cytoplasm and exhibited no apparent enhanced plasma membrane localization (Fig. 1, *A* and *C*; *control*). However, upon agonist-activation of the β_2AR, a time-dependent rapid redistribution of βarr_2GFP to the plasma membrane occurred (Fig. 1, *A* and *B*). The time course of β-arrestin translocation determined here, $t_{1/2}$ = 2.3 min (Fig. 1*B*), followed β_2AR phosphorylation ($t_{1/2}$ = 15–40 s) (24, 25) and preceded β_2AR internalization ($t_{1/2}$ = 10 min) (4). At first βarr_2GFP appeared diffusely at the plasma membrane, but with time a punctate pattern became apparent. Moreover, the redistribution of βarr_2GFP from the cytosol to the plasma membrane was agonist dose-dependent (Fig. 1, *C* and *D*). The half-maximal effective concentration (EC_{50}) of agonist was calculated to be 6 nM (Fig. 1*D*), a value comparable to that reported for β_2AR sequestration in HEK 293 cells (11 nM) (24). No significant βarr_2GFP translocation in response to agonist exposure was observed in cells lacking overexpressed β_2AR (data not shown). To further test the agonist specificity of βarr_2GFP translocation, cells were treated 5 min with 1 μM isoproterenol to induce βarr_2GFP translocation and then exposed to a saturating concentration of the antagonist propranolol in the presence of the agonist (Fig. 1*E*). Following the treatment of cells with the antagonist, the distribution of βarr_2GFP fluorescence reversed, with βarr_2GFP redistributing over time from the plasma membrane back to the cytoplasm. However, the redistribution of βarr_2GFP back into the cytoplasm was not immediate, but proceeded over a time course of 5–10 min consistent with previous reports describing agonist-dependent and independent steps of receptor internalization (26). Since the pharmacology of βarr_2GFP translocation accurately reflected the agonist dependence of β_2AR sequestration, these results suggest that β-arrestin translocation and receptor binding serve as the agonist-dependent switch triggering endocytosis of the β_2AR.

While βarr_2GFP was observed to translocate to the plasma

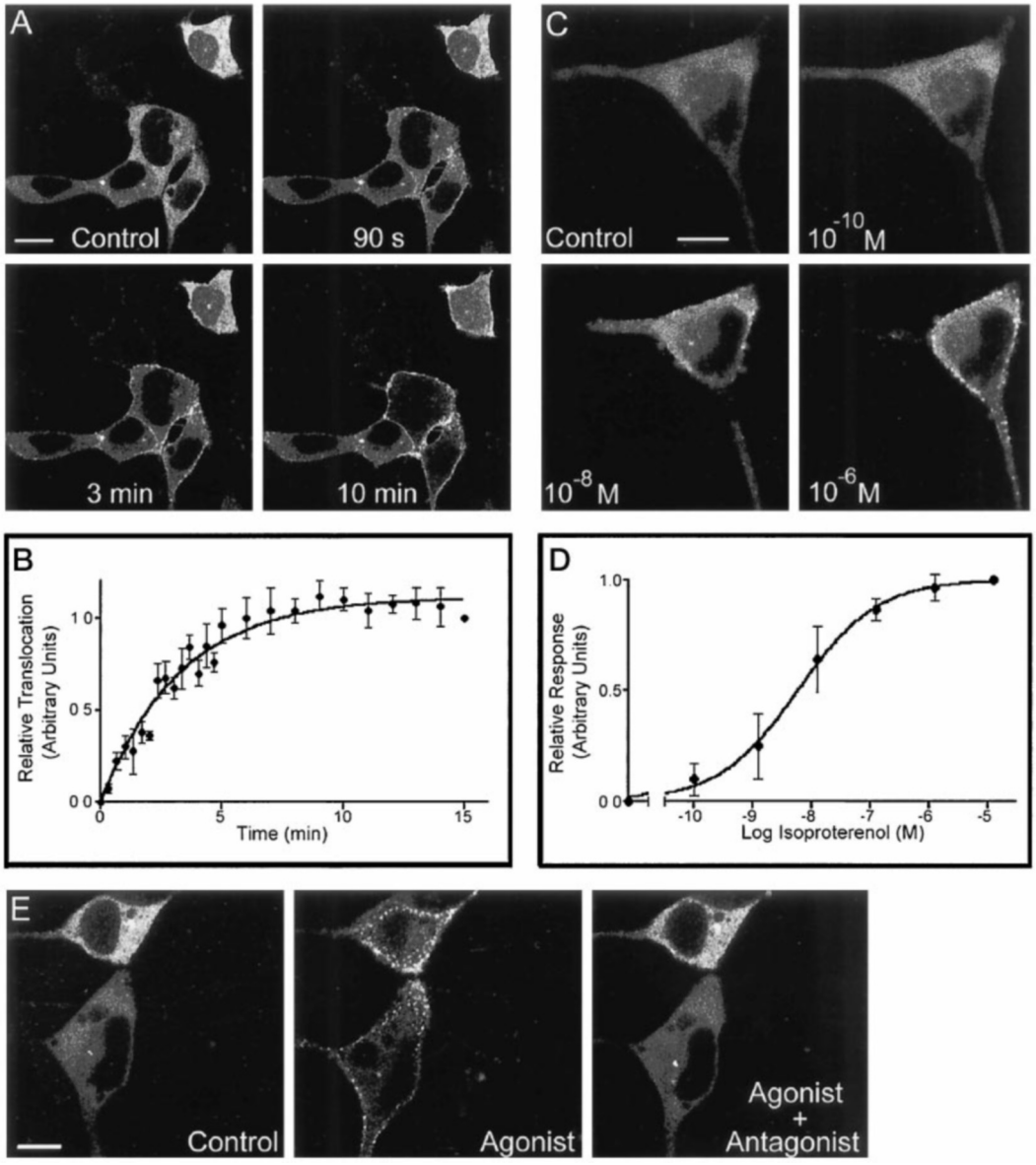

FIG. 1. **Pharmacology and agonist dependence of βarr$_2$GFP translocation in response to β_2AR activation in HEK 293 cells.** Visualization (*A*) and quantitation (*B*) of the time course for βarr$_2$GFP membrane translocation in HEK 293 cells expressing β_2AR and βarr$_2$GFP in response to stimulation with 25 μM isoproterenol for 0–15 min. Shown are representative confocal microscopic images of βarr$_2$GFP fluorescence obtained prior to (control) and 90 s, 3 min, and 10 min following the addition of agonist to the medium. Visualization (*C*) and quantitation (*D*) of the agonist dose-dependent membrane translocation of βarr$_2$GFP in HEK 293 cells in response to 5-min exposures to increasing concentration of isoproterenol 10^{-10} to 10^{-5} M. Shown are representative confocal microscopic images of βarr$_2$GFP fluorescence in HEK 293 cells obtained prior to (control) and following the addition of 10^{-10} M, 10^{-8} M, and 10^{-6} M isoproterenol. (*E*) Effect of treating cells with the antagonist propranolol on the localization of βarr$_2$GFP redistributed to the plasma membrane in response to receptor activation. Shown are representative confocal microscopic images of βarr$_2$GFP fluorescence in HEK 293 cells prior to (control) treatment for 5 min with 1 μM isoproterenol (agonist), following which antagonist (300 μM propranolol) was added to the agonist containing medium to compete for receptor binding sites containing medium and time scanned for an additional 10 min (agonist + antagonist). All cells were transfected with 1 μg of pGFP-N3/βarr2 and 5 μg of 12CA5 epitope-tagged β_2AR in pcDNA1-Amp, and experiments were performed independently on 4–8 different cells. Experiments were performed on a heated microscope stage set at 30 °C. Data points represent the mean ± S.D. of 8 (*B*) and 5 (*D*) different cells from separate transfections. Increased membrane localized fluorescence was quantitated using IPLab spectrum image analysis software (Signal Analytics Corp.). The *inset bars* represent 10 μm.

membrane and cluster at coated pits (18), βarr$_2$GFP labeling of intracellular endocytic vesicles following β_2AR activation was never observed. The overall distribution pattern of βarr$_2$GFP appeared different from that of a GFP-conjugated β_2AR (23) (Fig. 2, *A* and *B*). In response to agonist stimulation, the β_2AR-GFP redistributed from a diffuse plasma membrane localization to a membrane-associated vesiculated pattern, followed by the appearance of β_2AR-GFP in endocytic vesicles randomly distributed throughout the cytosol of the cell (Fig. 2*B*). Therefore, we examined the agonist-induced intracellular trafficking of both the β_2AR and βarr$_2$GFP in the same living cells. To do this, β_2ARs engineered with an amino-terminal HA epitope tag were expressed in HEK 293 cells with βarr$_2$GFP and labeled with 12CA5 monoclonal antibodies, which were themselves labeled with rhodamine-conjugated anti- Fabs. β_2ARs labeled in this manner were still able to respond normally to agonist activation (Fig. 2*C*). In the absence of agonist, β_2AR immunofluorescence (*red*) was localized solely to the plasma membrane, whereas βarr$_2$GFP fluorescence (green) was limited to the cytoplasm (Fig. 2*C*). In response to agonist activation of β_2ARs, βarr$_2$GFP translocated to the receptors at the plasma membrane. This was followed by the redistribution of both the

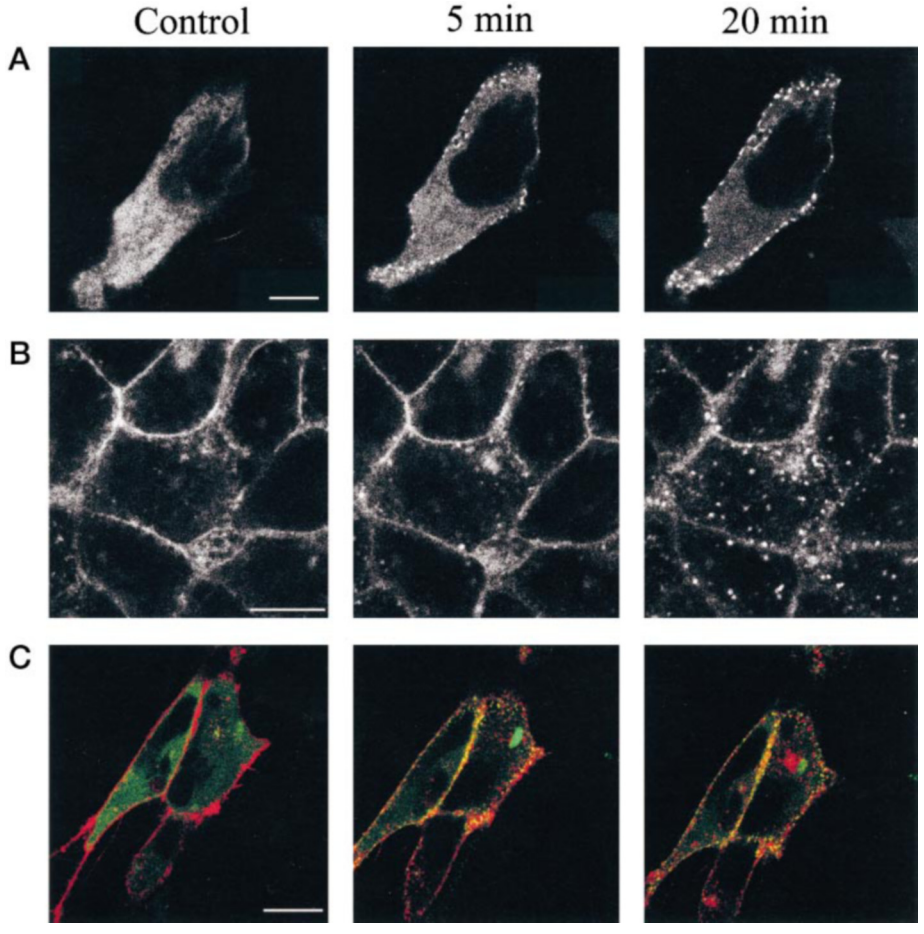

FIG. 2. **Redistribution and colocalization of the β_2AR and βarr_2GFP following agonist stimulation.** *A*, visualization of the redistribution of βarr_2GFP membrane fluorescence with time in response to the activation of the β_2AR with 25 μM isoproterenol. HEK 293 cells were transfected with 1 μg of pGFP-N3/βarr2 and 5 μg of 12CA5 epitope-tagged β_2AR in pcDNA1-Amp. Shown are representative confocal microscopic images of βarr_2GFP fluorescence in the same HEK 293 cells exposed to agonist for the times indicated. *B*, visualization of the redistribution of β_2AR-GFP membrane fluorescence with time in response to the activation of the β_2AR. HEK 293 cells were permanently transfected to express the 12CA5 epitope-tagged β_2AR-GFP construct (1.6 pmol/mg of protein) (23). Shown are representative confocal microscopic images of β_2AR-GFP fluorescence obtained prior (control) to the exposure of the same field of cells to 10 μM isoproterenol for 5 min and 20 min. *C*, confocal visualization of the intracellular distribution and colocalization (*yellow*) of βarr_2GFP (*green*) with 12CA5 epitope-tagged β_2ARs (*red*) labeled at 37 °C with 12CA5 monoclonal antibody followed by labeling with rhodamine-conjugated anti-rabbit Fabs at 4 °C in HEK 293 cells. Shown are representative confocal microscopic images of β_2AR and βarr_2GFP distribution prior to (control) and following the β_2AR activation with 25 μM isoproterenol at 30 °C for 5 and 20 min. Cells were transfected with 1 μg of pGFP-N3/βarr2 and 5 μg of 12CA5 epitope-tagged β_2AR in pcDNA1-Amp. All experiments were performed at 30 °C on three to six different occasions. The *inset bars* represent 10 μm.

receptors and β-arrestin to clathrin-coated pits, as denoted by the appearance of *yellow* hot spots (Fig. 2*C*). However, while *yellow* vesicles could be observed close to the membrane surface, no colocalization of βarr_2GFP with β_2AR-bearing vesicles was ever observed in the cytoplasm of the cell (Fig. 2*C*). A similar agonist-mediated redistribution of βarr_1GFP to plasma membrane-localized β_2AR, but not β_2AR localized in endocytic vesicles, was also observed (data not shown). These results demonstrate that β_2AR/β-arrestin complex dissociates at or close to the plasma membrane, and therefore β-arrestins are excluded from endocytic vesicles shortly following their formation.

In a previous study, we have demonstrated that although the $AT_{1A}R$ can utilize a distinct endocytic mechanism, overexpression of exogenous β-arrestins mobilizes the receptor for internalization via clathrin-mediated endocytosis similar to that utilized by the β_2AR (16). To further characterize the interaction of β-arrestin with the $AT_{1A}R$, receptor-mediated βarr_2GFP trafficking was examined in HEK 293 cells co-expressing the $AT_{1A}R$ and βarr_2GFP. The cells were stimulated with angiotensin II for various periods of time at 30 °C and observed under confocal microscope. Similar to that observed with the β_2AR, βarr_2GFP was evenly distributed throughout the cytoplasm in the absence of agonist, but underwent a rapid translocation to the plasma membrane in response to $AT_{1A}R$ activation (Fig. 3*A*). However, activation of the $AT_{1A}R$ for a longer period of time (>4 min) resulted in a clear redistribution of βarr_2GFP to intracellular endocytic vesicles. βarr_1GFP was also observed to undergo a similar redistribution to $AT_{1A}R$-containing endocytic vesicles.[2] With time, the βarr_2GFP-containing vesicular structures grew in size and were mobilized to cluster at the perinuclear region of the cells (Fig. 3*A*). In contrast, under parallel conditions, βarr_2GFP remained confined to the plasma membrane even when the β_2AR was activated by isoproterenol for 1 h (Fig. 3*B*). To further confirm that the redistribution of β-arrestins is receptor-driven, we examined the localization of agonist-activated $AT_{1A}R$ and βarr_2GFP in the same HEK 293 cells. To do this, $AT_{1A}R$s engineered with an amino-terminal HA epitope tag were expressed in HEK 293 cells with βarr_2GFP and labeled with rhodamine-conjugated anti-HA 12CA5 monoclonal antibodies. When the cells were

[2] R. Oakley, personal communication.

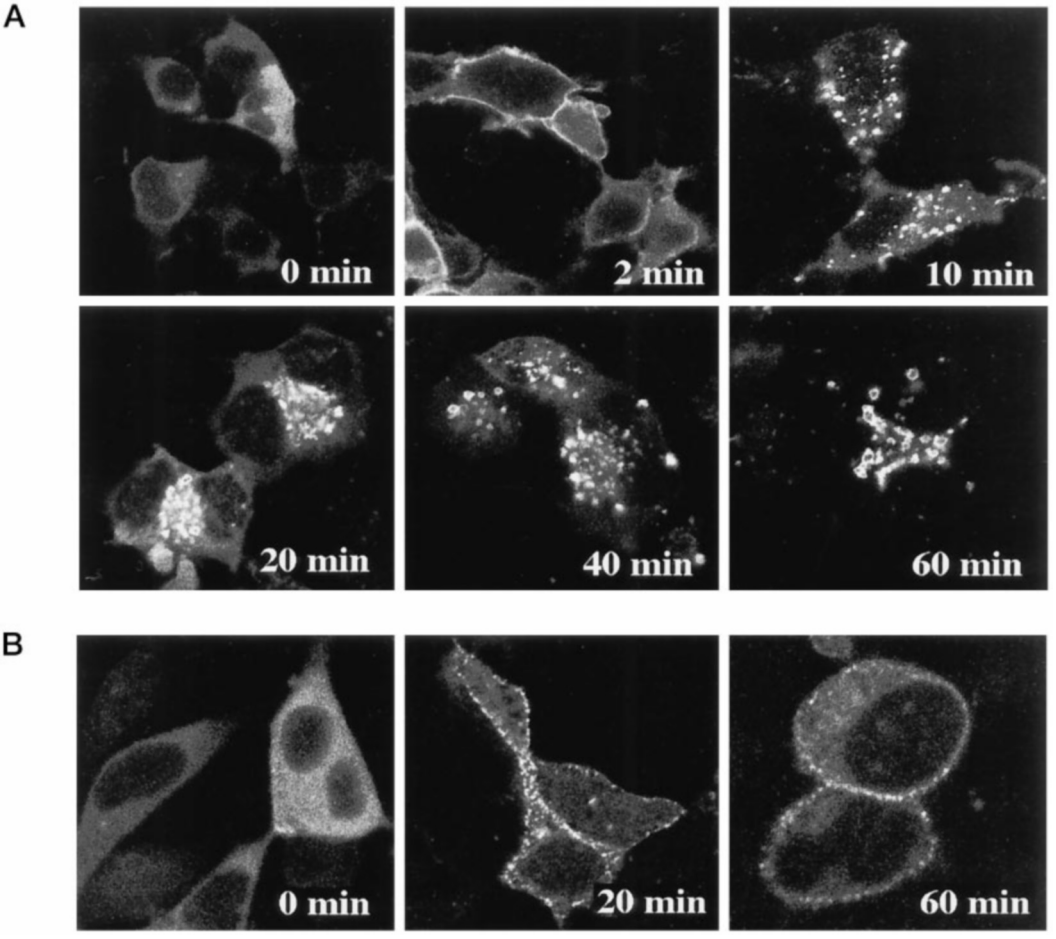

FIG. 3. **Differential cellular trafficking of βarr$_2$GFP in response to activation of the AT$_{1A}$R and β$_2$AR.** Visualization of the redistribution of βarr$_2$GFP fluorescence with time in response to the activation of the AT$_{1A}$R with 500 nM angiotensin II (*A*) or the β$_2$AR with 25 μM isoproterenol (*B*) for 0–60 min. HEK 293 cells were transfected with 1 μg of pGFP-N3/βarr2 and 5 μg of 12CA5 epitope-tagged AT$_{1A}$R or β$_2$AR in pcDNA1-Amp. Shown are representative confocal microscopic images of βarr$_2$GFP fluorescence in fixed HEK 293 cells exposed to agonist for the times indicated. All experiments were performed at 30 °C on three to five different occasions.

activated by angiotensin II, an agonist-dependent colocalization of AT$_{1A}$R red immunofluorescence and βarr$_2$GFP green fluorescence was observed and persisted for up to 1 h, as reflected by the predominant intracellular *yellow* vesicular structures located at the perinuclear region (Fig. 4). These data demonstrate a functional interaction between the AT$_{1A}$R and β-arrestin proteins. In addition, our results also indicate that, unlike the β$_2$AR/β-arrestin complex, the AT$_{1A}$R/β-arrestin complex remains intact and is mobilized to the interior of the cell driven by receptor internalization.

Since the activation of β$_2$AR and AT$_{1A}$R promoted β-arrestin trafficking to distinct subcellular locations, we examined the redistribution of βarr$_2$GFP in response to activation of several other GPCRs to address the generality of the two different β-arrestin translocation patterns. To do this, βarr$_2$GFP was co-expressed in HEK 293 cells with different GPCRs including dopamine D1A receptor (D$_{1A}$R), endothelin type A receptor (ET$_A$R) and neurotensin receptor (NTR). As shown in Fig. 5, in response to activation of the D$_{1A}$R and the ET$_A$R, βarr$_2$GFP underwent a rapid membrane translocation and remained in a punctate pattern at the periphery of the cells for as long as 1 h, similar to that observed following β$_2$AR stimulation. In contrast, in HEK 293 cells overexpressing the NTR, neurotensin stimulation resulted in the redistribution of βarr$_2$GFP fluorescence to intracellular vesicular structures with a pattern reminiscent of that observed following AT$_{1A}$R activation. Therefore, different GPCRs either separate from β-arrestins at the level of plasma membrane or internalize with β-arrestin in intracellular vesicles. This property appears independent of both the types of G protein-coupling and agonists.

Previous studies of GPCR internalization have suggested that the carboxyl-terminal region is important for receptor interaction with β-arrestins (4). Therefore, to examine whether the carboxyl-terminal tail of the receptors contributes to the differential trafficking of β-arrestin, we engineered chimeric mutants of the AT$_{1A}$R and β$_2$AR with their carboxyl-terminal region exchanged for that of the other. Like the wild-type AT$_{1A}$R and β$_2$AR, the chimeric AT$_{1A}$R and β$_2$AR mutants (namely AT$_{1A}$R-β$_2$AR-CT and β$_2$AR-AT$_{1A}$R-CT) underwent rapid internalization in response to agonist stimulation (Fig. 6*B*). However, when co-expressed with βarr$_2$GFP, angiotensin II activation of the AT$_{1A}$R-β$_2$AR-CT did not result in β-arrestin trafficking to intracellular vesicles, but rather resulted in a β-arrestin membrane localization pattern similar to that observed in response to β$_2$AR activation (Fig. 6*A*). In fact, βarr$_2$GFP fluorescence was retained at the plasma membrane for up to 1 h and was never localized to intracellular vesicular structures in response to activation of the chimeric AT$_{1A}$R mutant. In contrast, the corresponding β$_2$AR chimeric mutant with AT$_{1A}$R carboxyl-tail displayed "AT$_{1A}$R-like" phenotype and acquired the ability to mediate β-arrestin translocation to intracellular vesicles (Fig. 6*A*). In addition, a β$_2$AR-βarr$_2$GFP fusion protein with βarr$_2$GFP attached to the carboxyl terminus of the β$_2$AR was also localized to large intracellular vesic-

FIG. 4. **Colocalization of the $AT_{1A}R$ and βarr_2GFP following stimulation by angiotensin II.** Confocal visualization of the intracellular distribution and colocalization (overlay) of βarr_2GFP (*green*) with 12CA5 epitope-tagged $AT_{1A}Rs$ (*red*) labeled at 4 °C with rhodamine-conjugated 12CA5 monoclonal antibody in HEK 293 cells. Cells were transfected with 1 μg of pGFP-N3/βarr2 and 5 μg of 12CA5 epitope-tagged $AT_{1A}R$ in pcDNA1-Amp. Shown are representative confocal microscopic images of $AT_{1A}R$ and βarr_2GFP distribution following $AT_{1A}R$ activation with 500 nM angiotensin II at 30 °C for 1 h. The experiment was performed on two different occasions.

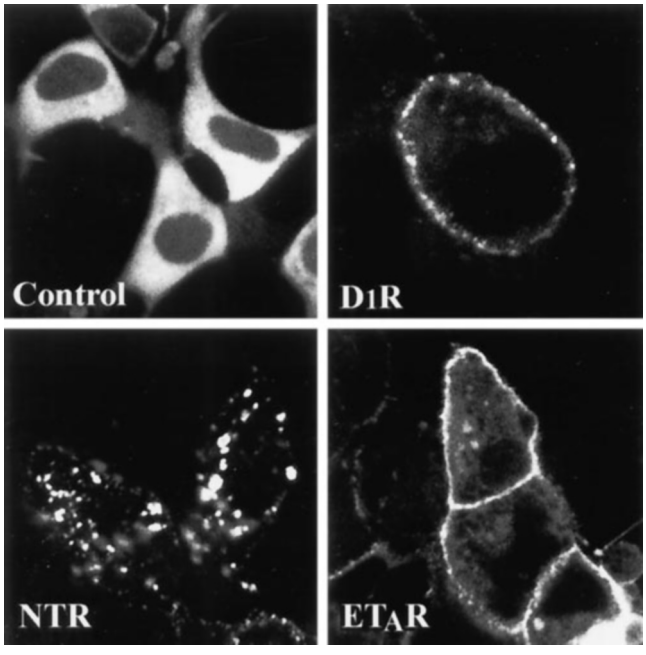

FIG. 5. **Translocation of βarr_2GFP in response to activation of the $D_{1A}R$, ET_AR, and NTR.** Visualization of the redistribution of βarr_2GFP fluorescence in response to activation of $D_{1A}R$ with 10 μM dopamine, ET_AR with 100 nM endothelin, or NTR with 1 μM neurotensin. HEK 293 cells were transfected with 1 μg of pGFP-N3/βarr2 and 5 μg of 12CA5 epitope-tagged $D_{1A}R$ in pcDNA1-Amp, ET_AR in pcDNA1, or NTR in pcDNA3. Shown are representative confocal microscopic images of βarr_2GFP fluorescence in fixed HEK 293 cells exposed to agonist for 60 min. All experiments were performed at 30 °C on three different occasions.

ular structures similar to the vesicular population containing βarr_2GFP following $AT_{1A}R$ stimulation (Fig. 6*C*). These results demonstrate that the carboxyl-terminal region of the receptors is involved in determining the mode of interaction of the receptors with β-arrestin proteins, and subsequently the agonist-dependent redistribution of β-arrestins.

DISCUSSION

In the present work, we use green fluorescent protein-conjugated β-arrestin2 to study the cellular trafficking of β-arrestin proteins in response to several different GPCRs in both living and fixed cells. By doing so, we show that the pharmacology of β-arrestin translocation underlies the inherent agonist dependence of β_2AR endocytosis. More interestingly, our results demonstrate that the cellular fate of β-arrestin2 (or β-arrestin1) is differentially regulated following activation of distinct GPCRs. A popular assumption in the field has been that β-arrestins are associated with internalized receptors in intracellular vesicles (reviewed in Refs. 21, 27, and 28). While this appears to be true for some receptors such as the $AT_{1A}R$ and NTR, we found that in the case of the β_2AR, $D_{1A}R$, and ET_AR, β-arrestins do not traffic with the receptors to endosomes and appear to dissociate from receptor-bearing vesicles shortly following their formation. In addition, swapping of the carboxyl-terminal regions between the $AT_{1A}R$ and β_2AR switches the phenotype of both receptors in terms of their ability to mobilize plasma membrane-associated β-arrestin to cytosolic vesicular structures. This indicates that the carboxyl-terminal region of the receptors is important in determining receptor/β-arrestin association and receptor-mediated β-arrestin cellular distribution to the plasma membrane and/or endosomes.

Considerable effort has been expended to uncover receptor endocytic motifs underlying the agonist-dependent endocytosis of GPCRs. The expectation was that discrete amino acid motifs on the GPCRs, similar in function to those utilized by single transmembrane spanning receptors, would be identified (21, 29, 30). However, the matching pharmacology for β-arrestin translocation and β_2AR sequestration described here strongly indicates that β-arrestin binding replaces the exposure of discrete amino acid motifs as the agonist-driven switch regulating receptor endocytosis. In contrast, the agonist dependence of the endocytosis of receptor tyrosine kinases is thought to involve the exposure of tyrosine-containing motifs on the receptors (30–34).

It is likely that the effect of agonist binding to the receptor is to induce an intramolecular rearrangement of multiple intracellular GPCR domains. This results in a generalized receptor conformation that is necessary to promote GRK phosphorylation and β-arrestin binding. Interestingly, this conformational requirement may account for the failure of some opioid agonists to induce the sequestration of the mu opioid receptor and the sequestration defect described for the β_2AR-Y326A mutant (11, 24, 35). Furthermore, experiments using β_2AR/β_3AR chimeric receptors showed that normal sequestration of the resulting chimeras required the swapping of several intracellular domains, including the first and second intracellular loops and the carboxyl tail between the two receptor proteins (36). Thus, it would appear that the endocytic switching function of β-arrestins is related to their role in receptor desensitization, *i.e.* the binding to and uncoupling of the receptor from its G protein. Once receptors are bound to β-arrestin, they then gain the ability to traffic to clathrin-coated pits. Indeed, photobleaching experiments of β_2AR-GFP in HEK 293 cells suggest that desensitized receptors (*i.e.* complexed with β-arrestins) are free moving in the plasma membrane and that their movement to coated pits is not rate-limiting for β_2AR endocytosis (23). This suggests that the interaction of β-arrestin with the β_2AR represents the initiating event for receptor endocytosis.

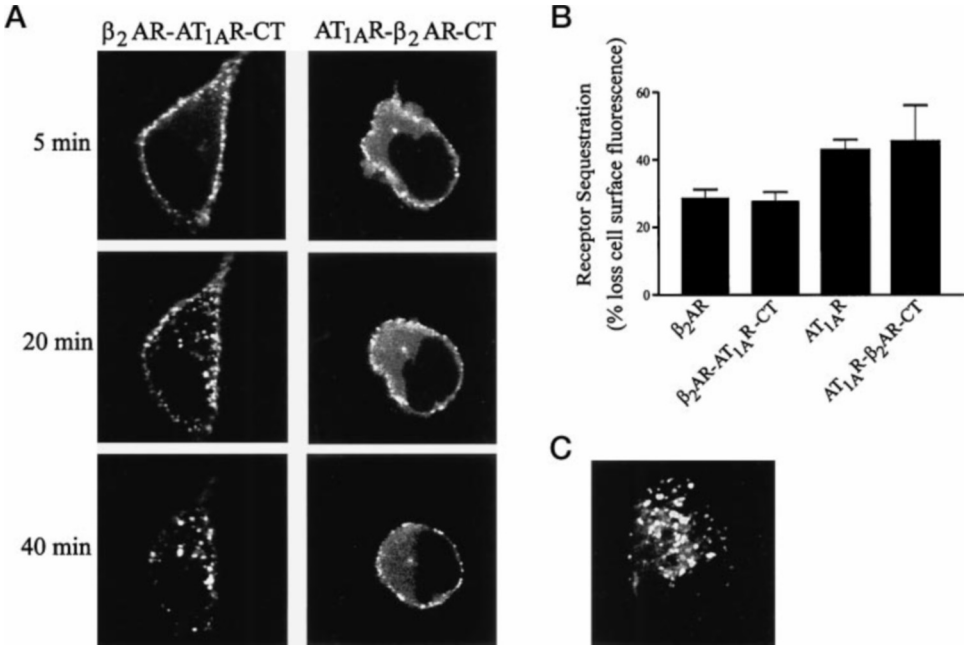

FIG. 6. **Effect of the carboxyl-terminal regions of the AT_{1A}R and β_2AR on βarr_2GFP localization.** *A*, visualization of the redistribution of βarr_2GFP fluorescence with time in response to stimulation of the AT_{1A}R-β_2AR-CT with 500 nM angiotensin II or the β_2AR-AT_{1A}R-CT with 10 μM isoproterenol. HEK 293 cells were transfected with 1 μg of pGFP-N3/$\beta arr2$ and 5 μg of AT_{1A}R-β_2AR-CT or β_2AR-AT_{1A}R-CT in pcDNA3. Shown are representative confocal microscopic images of βarr_2GFP fluorescence in HEK 293 cells exposed to agonist for the times indicated. *B*, sequestration of the wild-type AT_{1A}R, β_2AR and the chimeric mutants AT_{1A}R-β_2AR-CT and β_2AR-AT_{1A}R-CT. In these experiments, HEK 293 cells were also transiently transfected with 1 μg of pGFP-N3/βarr_2 and with plasmids containing cDNAs for HA epitope-tagged AT_{1A}R-β_2AR-CT and Flag epitope-tagged β_2AR-AT_{1A}R-CT. Receptor sequestration was assessed by flow cytometry as described previously (16). The data represent the mean ± S.E. of six experiments. *C*, cellular localization of a β_2AR-βarr_2GFP fusion protein. Cells were transfected with 5 μg of pGFP-β_2AR-βarr_2GFP. Shown are representative confocal microscopic images of β_2AR-βarr_2GFP distribution in HEK 293 cells.

Whereas there is growing evidence supporting β-arrestins as a general endocytic intermediate for many GPCRs, it is somewhat surprising that β-arrestins do not traffic to the same cellular compartments upon activation of distinct receptors. The internalization of β-arrestin with some GPCRs but not with others suggests that the properties of β-arrestin/receptor interactions differ for different GPCRs. For example, in the case of the α_2-adrenergic receptor, β-arrestins were demonstrated to bind to the third intracellular loop, whereas β-arrestin interactions with the β_2AR appear to involve multiple receptor domains including the receptor carboxyl terminus (36, 37). In addition, peptide inhibition studies suggest that the third and, to a lesser extent, the first intracellular loops of rhodopsin may play an important role in arrestin binding to light-activated forms of rhodopsin (38). In the present study, these differences are highlighted by the ability of β-arrestin2 to internalize with the AT_{1A}R and NTR, but not the β_2AR, D_{1A}R, and ET_AR. In the case of the AT_{1A}R, it appears that the carboxyl-terminal tail contributes directly to β-arrestin interactions. While it is plausible that these differences are the consequence of the high affinity and slow off-rate of peptidic ligands that might trap receptors in a conformation favoring stable β-arrestin binding, the observation that the ET_AR do not internalize with β-arrestin bound does not support this apparently simple explanation. Rather, the present experiments with an AT_{1A}R-β_2AR carboxyl-terminal tail chimera as well as a β_2AR-AT_{1A}R carboxyl-terminal tail chimera suggest that these differences appear to be regulated by differences in either the tertiary structure or β-arrestin-interacting sequences in the carboxyl-terminal domains of these receptors. Presumably, the carboxyl-terminal domain in conjunction with other intracellular receptor domains determines the relative stability of receptor/β-arrestin complexes.

In a previous study, we have reported that, although the AT_{1A}R is capable of utilizing a dynamin- and β-arrestin-independent endocytic pathway, co-expression of β-arrestins significantly increases the level of dynamin-dependent AT_{1A}R internalization (16). This suggests that the AT_{1A}R has the ability to directly interact with β-arrestins. In this study, using the GFP-conjugated β-arrestin2, we were able to visualize an agonist-dependent co-trafficking of the receptor with β-arrestins to endocytic vesicles. This represents the first direct demonstration of AT_{1A}R association with β-arrestins. As GRKs were shown to phosphorylate and desensitize the AT_{1A}R (39), it is probable that, similar to their role in β_2AR function, β-arrestins also play an important role in AT_{1A}R regulation by binding to the GRK-phosphorylated form of the receptor. In addition to the AT_{1A}R and β_2AR, we also visualized the trafficking of several other receptors with β-arrestins, including the D_1R, ET_AR, and NTR. The activation of the NTR resulted in an "AT_{1A}R-like" β-arrestin distribution pattern, whereas the activation of the other two receptors triggered a membrane localization of β-arrestins similar to that observed for the β_2AR. These observations indicate that, while GPCR/arrestin interactions represent a general GPCR regulatory mechanism, the stability of receptor/β-arrestin complexes differs from receptor to receptor.

The binding of arrestin proteins to GRK-phosphorylated GPCRs serves to desensitize various GPCRs, following which, the agonist-promoted receptor internalization is proposed to contribute to receptor dephosphorylation and resensitization (21, 40). A critical step leading to effective GPCR dephosphorylation and resensitization is the dissociation of the GPCR/arrestin complex, since dephosphorylation of rhodopsin was demonstrated to be blocked when the receptor is arrestin-bound (20). Our results indicate that β-arrestins dissociate from the β_2AR shortly following the redistribution of β-arrestins to coated pits. This early dissociation of GPCR/β-arrestin complexes is presumably appropriate to allow the β_2AR to associate with receptor phosphatase and dephosphorylate in

early endosomes (15). On the other hand, for GPCRs that internalize with β-arrestins bound, it might be expected that the kinetics of dephosphorylation of these receptors would be slower. While β-arrestin binding to receptors may be a general feature of GPCR regulation, our results suggest that the nature of this association or the stability of receptor/β-arrestin complex differs depending upon the receptor studied. Therefore, studies on the dissociation of receptor/β-arrestin complex should be valuable for understanding the mechanisms by which receptor desensitization and resensitization are achieved. The development of mutant or chimeric receptors with altered ability to interact with β-arrestins should greatly facilitate this goal.

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