Regulation of Angiotensin II Type 1A Receptor Intracellular Retention, Degradation, and Recycling by Rab5, Rab7, and Rab11 GTPases*

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Previous studies have demonstrated that the interaction of the angiotensin II type 1A receptor (AT1AR) carboxyl-terminal tail with Rab5α may modulate Rab5α activity, leading to the homotypic fusion of endocytic vesicles. Therefore, we have investigated whether AT1AR/Rab5α interactions mediate the retention of AT1ARβ-arrestin complexes in early endosomes and whether the overexpression of Rab7 and Rab11 GTPases influences AT1AR lysosomal degradation and plasma membrane recycling. We found that internalized AT1AR was retained in Rab5α-positive early endosomes and was neither targeted to lysosomes nor recycled back to the cell surface, whereas a mutant defective in Rab5a binding, AT1AR (1–349), was targeted to lysosomes for degradation. However, the loss of Rab5α binding to the AT1AR carboxyl-terminal tail did not promote AT1AR recycling. Rather, it was the stable binding of β-arrestin to the AT1AR that prevented, at least in part, AT1AR recycling. The overexpression of wild-type Rab7 and Rab7-Q67L resulted in both increased AT1AR degradation and AT1AR targeting to lysosomes. The Rab7 expression-dependent transition of “putative” AT1ARβ-arrestin complexes to late endosomes was blocked by the expression of dominant-negative Rab5α-S34N. Rab11 overexpression established AT1AR recycling and promoted the redistribution of AT1ARβ-arrestin complexes from early to recycling endosomes. Taken together, our data suggest that Rab5, Rab7, and Rab11 work in concert with one another to regulate the intracellular trafficking patterns of the AT1AR.

The angiotensin II type 1A receptor (AT1AR) is a member of the G protein-coupled receptor (GPCR) superfamily, the largest family of integral membrane receptor proteins. The AT1AR is coupled via Gq to the stimulation of phospholipase Cβ, leading to increases in intracellular diacylglycerol and inositol 1,4,5-trisphosphate and the release of calcium from intracellular stores (1). Agonist activation of the AT1AR also leads to the desensitization of AT1AR second messenger responses and the removal of cell-surface AT1AR into the intracellular compartment of the cell (2–4). The agonist-stimulated desensitization and endocytosis of many GPCRs is initiated by GPCR kinase-mediated phosphorylation, followed by β-arrestin binding (5). Both β-arrestin-dependent and β-arrestin-independent mechanisms of AT1AR endocytosis have been reported; and as such, the precise mechanism(s) regulating AT1AR internalization remain unclear (6, 7).

The AT1AR is a member of a class of GPCRs that remain associated with β-arrestins following their endocytosis (4, 8). Internalized AT1AR is targeted to enlarged hollow core vesicular structures, but is neither dephosphorylated nor efficiently recycled back to the cell surface (4). The inability of the vasoressin V2 receptor to recycle back to the cell surface appears to be regulated, at least in part, by the stable formation of a complex between β-arrestin and the G protein receptor kinase-activated Rab5α carboxyl-terminal tail of the receptor (9). This has led to the hypothesis that the formation of stable GPCR-β-arrestin complexes in endosomes may prevent the GPCR recycling and resensitization (10). However, exceptions do exist because other GPCRs, such as the neurokinin-1 receptor, internalize in a complex with β-arrestin and are still efficiently recycled back to the plasma membrane (11, 12).

The internalization and trafficking of GPCRs between intracellular membrane compartments play a crucial role in regulating the overall balance of GPCR activity by governing whether GPCRs are recycled back to the cell surface or degraded in lysosomes. Although the sorting of GPCRs between distinct intracellular membrane organelles, including early, recycling, and late endosomes, may be regulated in part by receptor/β-arrestin interactions, there is evidence that components of the endocytic machinery may directly influence the GPCR trafficking between these intracellular compartments. For example, AT1ARs preferenially traffic to Rab5-positive endosomal structures as a consequence of the agonist-dependent formation of AT1AR-Rab5α protein complexes and AT1AR-stimulated Rab5α GTP binding (13). However, the final intracellular destination of the AT1AR internalized to the Rab5-positive early endosomal compartment remains less clear.

In this study, we examine 1) whether the association of Rab5α with the AT1AR carboxyl-terminal tail prevents the lysosomal degradation and/or plasma membrane recycling of the AT1AR and 2) whether the overexpression of Rab11 and Rab7 GTPases promotes AT1AR recycling and trafficking to late endosomes and lysosomes. We found that Rab5α binding to the...
carboxy-terminal tail protected the AT₁AR from lysosomal degrada-
tion, but did contribute to the regulation of AT₁AR recycling.
Moreover, Rab7 and Rab11 overexpression increased AT₁AR targeting to lysosomes and recycling to the plasma membrane, suggesting that the relative level of Rab GTPase expression may influence the intracellular trafficking patterns and fate of GPCRs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human embryonic kidney (HEK) 293 cells were provided by American Type Culture Collection. The tissue culture medium was from Invitrogen. Bovine serum albumin was obtained from Bioshop Canada, Inc. Anti-rabbit IgG antibody was anti-IgG-Sepharose beads (Sigma). 12CA5 was purchased from Roche Applied Science. Horseradish peroxidase-conjugated donkey anti-mouse IgG secondary antibody was diluted 1:2500 in wash buffer containing 3% milk. The membranes were rinsed with wash buffer, incubated with ECL Western blot detection reagents, and then exposed to film.

**Quantitative Receptor Degradation Assays**—Cell-surface FLAG epitope-tagged receptors were labeled for 1 h on ice with mouse anti-FLAG antibody diluted in HBSS. Cells were then washed and allowed to warm to 37 °C prior to treatment with and without 100 nM AngII for 90 min at 37 °C. Cells were subsequently fixed and permeabilized in 2% formaldehyde in HBSS and 0.1% saponin, washed, and incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody to measure to the total cellular complement of FLAG epitope-tagged receptors. The total cellular complement of the remaining internalized cell-surface-retrieved fluorophore was determined following a 20-min incubation of solubilized cells with the horseradish peroxidase substrate tetramethylbenzidine (Sigma). The reaction was stopped with 1 N HCl, and the absorbance of the supernatant at 450 nm was measured using a microplate luminescence reader. Receptor degradation is defined as the loss of horseradish peroxidase activity following agonist stimulation. Experiments were done in quadruplicate for each condition and were repeated at least six times.

**Rab7 Immunoblotting**—HA-Rab7 expression was confirmed by immunoblotting. In brief, 50 μg of protein from each of the cell lysates used for immunoprecipitation were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and subsequently immunoblotted for HA-Rab7 expression with a rabbit anti-HA monoclonal antibody (1:10,000 dilution).

**Live Cell Imaging with Confocal Microscopy**—Confocal microscopy was performed using a Zeiss LSM-510 META laser-scanning microscope with a Zeiss 100 × 1.4 NA oil immersion lens. The Zeiss LSM-510 META system produces cross-talk-free images of fluorescent proteins (GFP and YFP) with closely overlapping emission spectra. This spectral separation, in addition to the use of anti-HA monoclonal antibody (1:10,000 dilution) to eliminate channel bleed-through, ² HEK 293 cells expressing either FLA-
G- or HA-tagged AT₃R and AT₃R(1–349) together with GFP- and YFP-tagged Rab and β-arrestin constructs as described in the figure legends were plated on 35-mm glass-bottomed culture dishes, and live cell images were taken in the absence and presence of treatment with 100 nM AngII. Cells were loaded with 50 nM LysoTracker Red dye for 30 min at 37 °C following agonist stimulation to assess the co-localization of receptors in lysosomes. Co-localization studies of β-
arrestin-2-GFP and LysoTracker Red-labeled lysosomes were performed using dual excitation (488 and 543 nm) and emission (505–530 nm, GFP; and 590–610 nm, LysoTracker Red) filter sets. The specificity of labeling and the absence of signal crossover were established by examination of single-labeled samples. Images showing λ scans of YFP- and GFP-expressing cells were unmixed using Zeiss LSM-510 META image processing software. ² For live cell receptor labeling, anti-FLAG monoclonal antibody was conjugated to either Alexa Fluor 488 or Alexa Fluor 555 using a Zenon mouse IgG labeling kit (Molecular Probes, Inc.) for co-localization studies of receptors in lysosomes. Cell-surface-retrieved fluorophore was determined following a 20-min incubation of solubilized cells with the horseradish peroxidase substrate tetramethylbenzidine (Sigma). The reaction was stopped with 1 N HCl, and the absorbance of the supernatant at 450 nm was measured using a microplate luminescence reader. Receptor degradation is defined as the loss of horseradish peroxidase activity following agonist stimulation. Experiments were done in quadruplicate for each condition and were repeated at least six times.

**RESULTS**

The AT₁AR Is Not Targeted to Lysosomes following Prolonged Agonist Treatment—Previous studies have demonstrated that the AT₁AR does not efficiently recycle to the cell surface and is internalized as a complex with β-arrestin to enlarged Rabβ-positive endocytic structures (4, 8, 13). Therefore, we sought to determine whether the AT₁AR is either retained in early endosomes or eventually targeted to lysosomes following prolonged agonist treatment. To examine AT₁AR targeting to lysosomes in live HEK 293 cells, we utilized the acido-tropic lysosomal compartment probe LysoTracker Red (16) in combination with β-arrestin-2-GFP to follow receptor trafficking (4, 8, 13). In the absence of agonist treatment, β-arrestin-2-GFP was diffusely localized throughout the cytoplasm, whereas LysoTracker Red stained punctu-

² Available at www.zeiss.com.
active vesicular structures within the cytosol of the cell (Fig. 1A). In response to a 3-h treatment of the same HEK 293 cell with 100 nM AngII, AT\textsubscript{1AR}-\beta-arrestin-2-GFP complexes internalized to large hollow core endosomal structures (Fig. 1B). Consistent with a previous study (13), AT\textsubscript{1A}R-\beta-arrestin-2-GFP complexes extensively co-localized with GFP-Rab5a (Fig. 1C). However, even following 3 h of agonist stimulation, the AT\textsubscript{1A}R-\beta-arrestin complexes rarely co-localized with the lysosomal marker LysoTracker Red (Fig. 1B). Taken together, these observations indicate that internalized AT\textsubscript{1AR} is retained in a Rab5-positive early endosomal compartment and does not target to lysosomes for degradation.

Rab5 Binding to the AT\textsubscript{1A}R Carboxyl-terminal Tail Prevents AT\textsubscript{1A}R Degradation—Truncation of the AT\textsubscript{1A}R carboxyl-terminal tail to create the AT\textsubscript{1A}R-(1-349) mutant lacking the distal 10 amino acid residues prevents Rab5 binding, but does not alter the stability of AT\textsubscript{1A}R-\beta-arrestin complexes (13). Furthermore, the AT\textsubscript{1A}R-(1-349) mutant only partially co-localizes with Rab5a in endocytic vesicles (13). Therefore, we tested the possibility that lost AT\textsubscript{1A}R-(1-349) binding to Rab5 may result in the lysosomal targeting of the mutant receptor. Following a 90-min exposure to 100 nM AngII, we observed substantial co-localization of AT\textsubscript{1A}R-\beta-arrestin-2-GFP with LysoTracker Red, whereas we did not observe co-localization between GFP-Rab5a and LysoTracker Red (Fig. 2, A and B). Agonist treatment of AT\textsubscript{1A}R-expressing HEK 293 cells for 2 and 4 h did not promote the degradation of

The AT\textsubscript{1A}R (Fig. 3A). Moreover, only 14 ± 7% of the wild-type AT\textsubscript{1A}R was degraded following a 90-min exposure to 100 nM AngII, whereas 33 ± 8% of the total cellular complement of AT\textsubscript{1A}R-(1-349) was lost following a 90-min exposure to agonist (Fig. 3, B and C). Thus, the truncation of the AT\textsubscript{1A}R tail, which impairs Rab5 interactions, also results in the targeting of the AT\textsubscript{1A}R to lysosomes for degradation.

Rab7-dependent Regulation of AT\textsubscript{1A}R Targeting to and Degradation in Lysosomes—Because Rab7 may regulate intracellular trafficking of vesicular cargo to late endosomes and lysosomes (16), we tested whether Rab7 overexpression might increase AT\textsubscript{1A}R degradation. Following Rab7 overexpression, agonist treatment of AT\textsubscript{1A}R-expressing HEK 293 cells for 2 and 4 h promoted significant AT\textsubscript{1A}R degradation (Fig. 3A). We found that the overexpression of either wild-type Rab7 or Rab7-Q67L increased wild-type AT\textsubscript{1A}R degradation to 29 ± 5 and 37 ± 7%, respectively (Fig. 3B). However, increased AT\textsubscript{1A}R degradation in response to 100 nM AngII treatment for 90 min was not observed following the overexpression of the dominant-negative Rab7-N125I mutant (Fig. 3B). In contrast, the extent of AT\textsubscript{1A}R-(1-349) mutant degradation following 90 min of agonist treatment was unaffected by Rab7 and Rab7-Q67L expression, but dominant-negative Rab7-N125I reduced AT\textsubscript{1A}R-(1-349) degradation to 11 ± 7% of the total cellular complement of the receptor (Fig. 3C). The overexpression of either Rab7 or Rab7-Q67L resulted in the co-localization of “putative” wild-type AT\textsubscript{1A}R-\beta-arrestin complexes with the lysosomal marker dye LysoTracker Red (Fig. 4, A and B). Although the overexpression of Rab7-N125I had no effect on the localization of the wild-type AT\textsubscript{1A}R-\beta-arrestin complexes, it prevented the redistribution of the AT\textsubscript{1A}R-(1-349) mutant-\beta-arrestin complexes to LysoTracker Red-positive lysosomes (Fig. 4, C and D). We have demonstrated that the AT\textsubscript{1A}R remains associated with \beta-arrestin following the internalization of the receptor (8), but this does not rule out the possibility that only \beta-arrestin is targeted to lysosomes following Rab7 expression. Furthermore, Bhatnagar et al. (17) have reported previously that \beta-arrestin dissociates from the 5-hydroxytryptamine type 2c receptor in
the endosomal compartment. Therefore, we tested whether the AT1AR may be targeted to lysosomes following the prolonged treatment of cells with agonist. In the absence of agonist, AT1AR labeled with Alexa Fluor 488-conjugated anti-FLAG antibody was localized to the cell surface (Fig. 5A). Agonist treatment for 2 h resulted in the internalization of anti-FLAG antibody-labeled AT1AR to large hollow core vesicles, but the receptor did not co-localize with LysoTracker Red-positive lysosomes (Fig. 5B). However, identical to what was observed for AT1AR-β-arrestin complexes, the expression of both wild-type Rab7 and Rab7-Q79L resulted in the co-localization of wild-type AT1AR with LysoTracker Red (Fig. 5C and D). Thus, it is unlikely that the receptor and β-arrestin traffic to distinct compartments following Rab7 overexpression. Taken together, the data suggest that, although the AT1AR is normally retained within a Rab5-positive early endosomal compartment, increased Rab7 activity promotes the transition of the AT1AR from the early endosomal compartment to lysosomes.

Role of Rab7 and Rab5 in Regulating the Targeting of the AT1AR to Late Endosomes—To determine how Rab7 overexpression might facilitate the lysosomal targeting of the AT1AR in HEK 293 cells, we examined whether the subcellular distribution of Rab7 overlaps with either LysoTracker Red in lysosomes or Rab5 in early endosomes. We found that, although YFP-Rab7 exhibited extensive co-localization with LysoTracker Red-positive lysosomes (Fig. 6A, yellow), YFP-Rab7 also labeled an endosomal compartment that was not LysoTracker Red-positive (green). In addition, we observed LysoTracker Red-positive lysosomes that did not contain YFP-Rab7 (Fig. 6A, red). Although GFP-Rab5a and YFP-Rab7 primarily labeled distinct membrane compartments, there appears to be

**FIG. 3.** Effect of wild-type and mutant Rab7 expression on AT1AR and AT1AR-(1–349) degradation. Shown in A is a representative immunoblot of co-immunoprecipitated FLAG-tagged AT1AR (upper panel) prior to and following 100 nM AngII treatment for 2 and 4 h in the presence and absence of HA-Rab7 overexpression (lower panel). Data shown are representative of three independent experiments. The histograms show the effect of Rab7, Rab7-Q67L, and Rab7-N125I overexpression on the degradation of HA-AT1AR (B) and HA-AT1AR-(1–349) (C) following treatment of HEK 293 cells for 90 min with 100 nM AngII. Data represent the means ± S.E. of six independent experiments. HEK 293 cells were transiently transfected with plasmid cDNAs encoding HA-AT1AR (10 μg), HA-AT1AR-(1–349) (10 μg), GFP-Rab7 (8 μg), GFP-Rab7-Q67L (8 μg), and GFP-Rab7-N125I (8 μg). *, p < 0.05 compared with control AT1AR and AT1AR-(1–349) degradation. NT, not treated.

**FIG. 4.** Co-localization of AT1AR-β-arrestin complexes with LysoTracker Red following the overexpression of Rab7, Rab7-Q67L, and Rab7-N125I. Shown are representative laser-scanning confocal micrographs of the effect of wild-type Rab7 (A), Rab7-Q67L (B), and Rab7-N125I (C) overexpression on the distribution of AT1AR-β-arrestin-2-GFP complexes (green) and LysoTracker Red (red) in live HEK 293 cells treated with 100 nM AngII for 90 min. Also shown are representative laser-scanning confocal micrographs of the effect of Rab7-N125I on the distribution of AT1AR-(1–349)-β-arrestin-2-GFP complexes (green) and LysoTracker Red (red) in live HEK 293 cells treated with 100 nM AngII for 90 min (D). Yellow indicates co-localization. Data are representative images of multiple cells from four independent experiments. HEK 293 cells were transiently transfected with plasmid cDNAs encoding HA-AT1AR (10 μg), HA-AT1AR-(1–349) (10 μg), β-arrestin-2-GFP (5 μg), HA-Rab7 (10 μg), HA-Rab7-Q67L (10 μg), and HA-Rab7-N125I (10 μg). Bars = 10 μm.
some overlap in GFP-Rab5a and YFP-Rab7 in HEK 293 cells (Fig. 6B, arrows). Consistent with a role for Rab7 in regulating vesicular trafficking from early to late endosomes, internalized AT1AR-β-arrestin-2-GFP complexes became extensively co-localized with overexpressed YFP-Rab7 (Fig. 7A, arrows). Similarly, Alexa Fluor 555-conjugated anti-FLAG antibody-labeled AT1AR was also targeted to YFP-Rab7-positive vesicles (Fig. 7B). The overexpression of the dominant-negative Rab5a-S34N mutant prevented both the co-localization of AT1AR-β-arrestin-2-GFP complexes with YFP-Rab7 and the targeting of the AT1AR-(1–349) mutant to lysosomes (Fig. 7, C and D). These results support previous observations that both Rab5 and Rab7 regulate the trafficking of cargo proteins from early endosomes to late endosomes and lysosomes (16, 18). Thus, we propose that increased Rab7 activity promotes the exit of AT1AR from early endosomes, ultimately allowing AT1AR degradation in lysosomes.

AT1AR Plasma Membrane Recycling—The AT1AR is not efficiently recycled back to the cell surface following agonist removal (4). Therefore, we investigated the possibility that loss of Rab5 binding to the AT1AR carboxyl-terminal tail might promote AT1AR recycling. However, we found that neither the AT1AR-(1–349) mutant nor the wild-type AT1AR was effectively recycled (16 ± 5 and 20 ± 5% cell-surface recovery, respectively), whereas 77 ± 7% of the β2-adrenergic receptor (β2AR) was efficiently recycled back to the cell surface 1 h following agonist removal (Fig. 8, A–C). In contrast, the AT1AR-AALAA mutant (10), which lacks serine and threonine residues required for stable β-arrestin binding, recycled more efficiently than the wild-type AT1AR, but less efficiently than the β2AR (Fig. 8D). Thus, the plasma membrane recycling of the AT1AR is prevented, at least in part, by the formation of stable receptor-β-arrestin complexes in endosomes.

Rab11-dependent Regulation of AT1AR Plasma Membrane Recycling—Because Rab7 overexpression influenced the targeting of the AT1AR to lysosomes, we sought to determine whether wild-type, constitutively active (Q70L), and dominant-negative (S25N) Rab11 protein overexpression might alter the plasma membrane recycling of the β2AR and AT1AR. We found that the plasma membrane recycling of the β2AR was unaffected by either wild-type Rab11 or Rab11-Q70L overexpression, whereas Rab11-S25N reduced β2AR recycling by 24 ± 4% (Fig. 8A). However, the overexpression of both the wild-type Rab11 and Rab11-Q70L proteins significantly increased AT1AR recycling to 61 ± 9 and 61 ± 10%, respectively (Fig. 8B). However, only Rab11-Q70L expression increased the plasma membrane recycling of the AT1AR-(1–349) and AT1AR-AALAA mutants (Fig. 8, C and D). Rab11-S25N did not alter the extent of membrane recycling for any of the AT1AR constructs (Fig. 8, B–D). Thus, similar to what we observed for AT1AR degradation following Rab7 overexpression, Rab11 overexpression facilitated AT1AR recycling.

Effect of Rab11 on the Subcellular Distribution of AT1AR-β-Arrestin Complexes—Consistent with previous observations that Rab5 and Rab11 are each localized to overlapping, yet distinct early endosomal domains (19), we observed the punctuate co-localization of GFP-Rab11 at the rim of endosomes containing AT1AR-β-arrestin-2-YFP complexes (Fig. 9A, arrows). In contrast, we found that GFP-Rab11-Q70L was extensively co-localized with AT1AR-β-arrestin-2-YFP complexes in enlarged endosomes (Fig. 9B, arrows). Because Rab7-Q70L...
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In this study, we have investigated whether the association of Rab5 with the AT1AR carboxyl-terminal tail results in the retention of the receptor in early endosomes, thereby preventing AT1AR recycling and degradation. We found that the wild-type AT1AR endocytosed to Rab5-positive endosomes was neither targeted to lysosomes nor recycled back to the cell surface, whereas the AT1AR-(1–349) mutant, which did not bind Rab5, was targeted to lysosomes for degradation. However, the loss of Rab5 binding to the AT1AR carboxyl-terminal tail did not re-establish AT1AR recycling. Rather, a loss of stable β-arrestin binding to the AT1AR, at least in part, allowed the AT1AR to recycle. Thus, Rab5 and β-arrestin binding to the AT1AR carboxyl-terminal tail appear to function together to mediate the retention of the AT1AR in early endosomes. We also observed that the transition of the wild-type AT1AR from early endosomes to late and recycling endosomal compartments was facilitated by the overexpression of Rab7 and Rab11 proteins, respectively. The targeting of the AT1AR to late endosomes and lysosomes was blocked by the expression of the dominant-negative Rab5a-S34N mutant. Taken together, our data suggest that Rab5, Rab7, and Rab11 have the potential to work in concert with one another to regulate the intracellular trafficking patterns of the AT1AR.

The transport of proteins between distinct intracellular organelles is a highly regulated process that involves multiple vesicular membrane budding and fusion events between donor and acceptor membranes. Rab GTPases are key players that regulate vesicular trafficking between these endosomal compartments (20–22). This study focused on the specific role of three Rab GTPases, Rab5, Rab7, and Rab11, in regulating the intracellular trafficking of the AT1AR. We have found that endocytosed AT1AR/β-arrestin complexes are normally retained in enlarged endosomal structures (4, 13), but that the overexpression of Rab7 and Rab11 GTPases promotes the redistribution of these putative complexes to late and recycling endosomal membrane compartments. The retention of the AT1AR in early endosomes is likely the result of AT1AR/Rab5 interactions, leading to increased Rab5 activity, and increased Rab7 and Rab11 activity may be sufficient to alter the trafficking of the receptor between membrane compartments. Thus, despite the fact that the AT1AR may regulate Rab5 activity and early endosomal fusion (13), Rab7 and Rab11 overexpression may overcome this activity to substantially increase AT1AR transit to late and recycling endosomal compartments. This conclusion is consistent with previous observations that the expression of the constitutively active Rab5-Q79L mutant increases the rate of transferrin uptake and results in the accumulation of the β2AR in enlarged Rab5-positive endosomes (15, 23). Consequently, despite the fact that Rab5, Rab7, and Rab11 GTPases are ubiquitously expressed, cell type and tissue differences in Rab GTPase protein expression levels may have profound effects on AT1AR trafficking between intracellular membrane compartments and the plasma membrane surface. Thus, the AT1AR may exhibit diverse desensitization and resensitization profiles in different cell types.

Many GPCRs, including the β2AR, 5-opioid receptor, κ-opioid receptor, endothelin B receptor, protease-activated receptor-1, CXCR chemokine receptor-2, and CXC chemokine receptor-4, are degraded in response to prolonged agonist stimulation (27–29). Consequently, the mechanisms contributing to the targeting of GPCRs for degradation in both proteosomes and lysosomes have become the subject of intensive investigation. The degradation of the β2AR and κ-opioid receptor is reported to be

overexpression increased the plasma membrane recycling of the AT1AR, AT1AR-(1–349), and AT1AR-ALAA, we examined the effect of Rab11-Q79L overexpression on the co-localization of internalized β-arrestin-2-YFP and Alexa Fluor 555-conjugated anti-FLAG antibody-labeled AT1AR with GFP-Rab5a. To our surprise, we found that Rab11-Q79L overexpression resulted in the loss of both β-arrestin-2-YFP and anti-FLAG antibody-labeled AT1AR co-localization with GFP-Rab5a (Fig. 9, C and D, arrows). Taken together, our observations suggest that the overexpression of both Rab7 and Rab11 GTPases not only results in the redistribution of the AT1AR to late and recycling endosomes, but also appears to dynamically regulate AT1AR/Rab5 interactions.

DISCUSSION

FIG. 7. Effect of Rab7 and dominant-negative Rab5a-S34N mutant expression on the redistribution of AT1AR/β-arrestin-2 complexes to late endosomes. A, representative laser-scanning confocal micrographs showing the co-localization of AT1AR/β-arrestin-2-GFP complexes (green) with YFP-Rab7 (red)-positive late endosomes following treatment of live HEK 293 cells with 100 nM AngII for 60 min. B, representative laser-scanning confocal images showing the co-localization of Alexa Fluor 555-conjugated anti-FLAG monoclonal antibody-labeled FLAG-AT1AR (100 nM AngII) with GFP-Rab5 (green) and of AT1AR/β-arrestin-2-GFP complexes (green) with YFP-Rab7 (red)-positive late endosomes following treatment of live HEK 293 cells with 100 nM AngII for 60 min. C, representative laser-scanning confocal micrographs showing the effect of Rab5a-S34N on the co-localization of AT1AR/β-arrestin-2-GFP complexes (green) with YFP-Rab7 (red)-positive late endosomes following treatment of live HEK 293 cells with 100 nM AngII for 60 min. D, representative laser-scanning confocal micrographs showing the effect of Rab5a-S34N on the co-localization of AT1AR/β-arrestin-2-GFP complexes (green) with LysoTracker Red (red) in lysosomes following treatment of live HEK 293 cells with 100 nM AngII for 60 min. Yellow indicates co-localization. Data are representative images of multiple cells from three to five independent experiments. HEK 293 cells were transiently transfected with plasmid cDNAs encoding FLAG-AT1AR (100 nM AngII), HA-AT1AR-(1–349) (10 μg), β-arrestin-2-GFP (5 μg), YFP-Rab7 (5 μg), and Rab5a-S34N (10 μg). Bars = 10 μm.
mediated by both lysosomes and proteasomes (24, 26, 30), whereas ubiquitin-dependent lysosomal degradation has been reported for CXC chemokine receptor-4 (28). β2AR have also been implicated in the ubiquitin-mediated down-regulation of the β2AR via association with Mdm2 (31). However, Mdm2 does not appear to be the same ubiquitin ligase that mediates β2AR ubiquitination (31). The targeting of protease-activated receptor-1 for lysosomal degradation is mediated by the association of sorting nexin-1 with the carboxyl-terminal tail of the receptor (32). Due to the localization of Rab7 to late endosomes and lysosomes, Rab7 is proposed to regulate vesicular trafficking from early endosomes to late endosomes and from late endosomes to lysosomes (16, 18). Thus, it is likely that, no matter which mechanism and/or molecular intermediate is required for the targeting of GPCRs to lysosomes, the receptors may be mobilized to lysosomes via Rab7-positive late endosomes. Consistent with this hypothesis, identical to what we observed for the AT1AR-(1–349) mutant, the overexpression of Rab11 or Rab11-Q70L increased the plasma membrane recycling of the AT1AR. This observation is consistent with a previous report suggesting that the AT1AR may recycle through the Rab11-mediated slow recycling route (40). However, only Rab11-Q70L stimulated the recycling of the AT1AR-(1–349) mutant. It is not clear why wild-type Rab11

![Fig. 8. Effect of wild-type and mutant Rab11 expression on AT1AR and AT1AR-(1–349) plasma membrane recycling.](image)

The histograms show the effects of Rab11, Rab7-Q70L and Rab11-S25N overexpression on the plasma membrane recycling of FLAG-β2AR (A), FLAG-AT1AR (B), FLAG-AT1AR-(1–349) (C), and FLAG-AT1AR-AALAA (D) following a 60-min recovery of HER 293 cells in agonist-free medium. HER 293 cells were treated with 100 nM AngII for 30 min prior to washing and recovery in agonist-free medium. Data represent the means ± S.E. of eight independent experiments. HER 293 cells were transiently transfected with plasmid cDNAs encoding FLAG-β2AR (10 μg), FLAG-AT1AR (10 μg), FLAG-AT1AR-(1–349) (10 μg), FLAG-AT1AR-AALAA (10 μg), HA-Rab11 (8 μg), HA-Rab11-Q70L (8 μg), and HA-Rab11-S25N (8 μg). *, p < 0.05 compared with control recycling values for each receptor.
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**Fig. 9. Effect of Rab11 on the co-localization of AT$_{1}$R-$\beta$-arrestin-2 complexes with Rab11- and Rab5-positive endosomal structures.** A, representative laser-scanning confocal micrographs showing the co-localization of GFP-Rab11 (green) with AT$_{1}$R-$\beta$-arrestin-2-YFP complexes (red) following treatment of live HEK 293 cells with 100 nM AngII for 60 min. B, representative laser-scanning confocal micrographs showing the co-localization of GFP-Rab11-Q70L (green) with AT$_{1}$R-$\beta$-arrestin-2-YFP complexes (red) following treatment of live HEK 293 cells with 100 nM AngII for 60 min. C, representative laser-scanning confocal micrographs showing the effect of Rab11-Q70L on the co-localization of GFP-Rab5a (green) with AT$_{1}$R-$\beta$-arrestin-2-YFP complexes (red) following treatment of live HEK 293 cells with 100 nM AngII for 60 min. D, representative laser-scanning confocal micrographs showing the effect of Rab11-Q70L on the co-localization of GFP-Rab5a (green) with Alexa Fluor 555-conjugated anti-FLAG monoclonal antibody-labeled FLAG-AT$_{1}$R (red) following treatment of live HEK 293 cells with 100 nM AngII for 60 min. Yellow indicates co-localization. Data are representative images of multiple cells from three to five independent experiments. HEK 293 cells were transiently transfected with plasmid cDNAs encoding FLAG-AT$_{1}$R (10 µg), β-arrestin-2-YFP (5 µg), GFP-Rab11 (3.5 µg), GFP-Rab11-Q70L (3.5 µg), GFP-Rab5a (5 µg), and HA-Rab11-Q70L (5 µg). Bars = 10 µm.

Regulates only wild-type AT$_{1}$AR recycling; it is possible that, similar to what we have observed for Rab5 (13), the activity of overexpressed Rab7 may also be regulated by the AT$_{1}$R and that β-arrestin binding may be required for this activity. This possibility warrants future investigation.

Recycling of the AT$_{1}$AR is increased following loss of β-arrestin binding, but not Rab8 interactions, supporting the idea that the formation of stable receptor-β-arrestin complexes may retard receptor recycling (10). However, the neurokinin-1 receptor internalizes bound to β-arrestin, but is efficiently recycled back to the cell surface (36). The overexpression of Rab11-Q70L not only promotes the redistribution ofputative AT$_{1}$R-β-arrestin complexes out of the Rab5-positive endosomes, but increases the recycling of wild-type and mutant AT$_{1}$ARs. Thus, it is possible that Rab protein expression may differentially regulate the trafficking of different GPCRs through distinct Rab-regulated endosomal compartments. The effect of increased Rab11 activity may be to overcome the β-arrestin-dependent inhibition of GPCR recycling.

In conclusion, it is now clear that the targeting of GPCRs between intracellular membrane compartments involves a complex series of protein/protein interactions, such as the binding of sorting nexin-1 to protease-activated receptor-1 and Rab5 to the AT$_{1}$R (13, 32). In addition, the targeting of proteins between intracellular compartments requires the coordinated regulation of vesicular trafficking by Rab GTPases. In this study, we have demonstrated that, by virtue of their ability either to bind to the AT$_{1}$R or to regulate vesicular trafficking to late and recycling endosomes, Rab GTPases interactively coordinate the intracellular trafficking fate of the AT$_{1}$R. Because >60 Rab GTPases may exist (21), it is likely that other Rab GTPases may regulate the trafficking of GPCRs between additional membrane compartments and/or that it will be possible to identify increasingly discrete membrane compartments that regulate the activation, inactivation, and reactivation of GPCRs. Future challenges will be to further characterize the precise roles of other Rab GTPases in the intracellular trafficking of the AT$_{1}$R and other GPCRs.
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