Published in final edited form as:

Cell Signal. 2011 January ; 23(1): 46–57. doi:10.1016/j.cellsig.2010.07.020.

Rab11a and its binding partners regulate the recycling of the β 1-adrenergic receptor

Lidia A. Gardner^{§,*}, Hassan Hajjhussein^{*}, Katherine C. Frederick, and Suleiman W. Bahouth[¶]

Department of Pharmacology, University of Tennessee Health Sciences Center, 874 Union Avenue, Memphis, TN 38163

[§]Department of Neurology, University of Tennessee Health Sciences Center, 874 Union Avenue, Memphis, TN 38163

Abstract

β 1-adrenergic receptors (β 1-AR) are internalized in response to agonists and then recycle back for another round of signaling. The serine 312 to alanine mutant of the β 1-AR (S312A) is internalized but does not recycle. We determined that WT β 1-AR and S312A were internalized initially to an early sorting compartment because they colocalized by >70% with the early endosomal markers rab5a and early endosomal antigen-1 (EEA1). Subsequently, the WT β 1-AR trafficked via rab4a-expressing sorting endosomes to recycling endosomes. In recycling endosomes WT β 1-AR were colocalized by >70% with the rab11 GTPase. S312A did not colocalize with either rab4a or rab11, instead they exited from early endosomes to late endosomes/lysosomes in which they were degraded. Rab11a played a prominent role in recycling of the WT β 1-AR because dominant negative rab11a inhibited, while constitutively active rab11a accelerated the recycling of the β 1-AR. Next, we determined the effect of each of the rab11-interacting proteins on trafficking of the WT β 1-AR. The recycling of the β 1-AR was markedly inhibited when myosin Vb, FIP2, FIP3 and rabphilin were knocked down. These data indicate that rab11a and a select group of its binding partners play a prominent role recycling of the human β 1-AR.

Keywords

β 1-adrenergic receptors; recycling; rab proteins; colocalization confocal microscopy; biotinylation

1. Introduction

Chronic activation of G protein-coupled receptors (GPCR) promotes the desensitization and uncoupling of the GPCR from the G protein. Chronic exposure of GPCR to agonists also induces the internalization of the GPCR via clathrin-coated vesicles into sealed endocytic compartments. These compartments either recycle the GPCR back to the membrane or are retained intracellularly where their contents are eventually degraded [1].

[¶]To whom correspondence should be addressed. Corresponding Author: Suleiman Bahouth, Ph.D., Department of Pharmacology, The University of Tennessee-HSC, 874 Union Avenue, Memphis, TN 38163, Tel: 901-448-1503, Fax: 901-448-7206, sbahouth@uthsc.edu.

^{*}These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

We have determined that recycling of the β 1-AR promotes its resensitization and reestablishes functional responsiveness of the β 1-AR towards its signaling pathway [2]. The domains involved in determining whether a given GPCR is recycled or retained within the cell are yet to be characterized. In this regard, two domains within the β 1-AR were responsible for its efficient recycling. The first domain was the type I PDZ “ESKV” sequence in the carboxy-terminus, which binds to a scaffold that targets PKA to the β 1-AR microdomain [3,4]. The second domain was the serine at position 312 in the 3rd intracellular loop of the β 1-AR, which is reversibly phosphorylated by PKA [3]. Consequently, mutagenesis of Ser312 to alanine (S312A) or inactivation of PKA both inhibited the recycling of the human β 1-AR [2]. The purpose of this study was to map the intracellular trafficking pathways of the recycling WT β 1-AR and the nonrecycling S312A β 1-AR in human embryonic kidney-293 (HEK-293) cells.

While intracellular trafficking of several GPCR has been partially determined, there have been no studies on the trafficking of the WT β 1-AR. In these studies, trafficking of a given GPCR was mapped by colocalization between the GPCR and trafficking rab proteins [5–8]. Rab proteins are small GTPases that recruit peripheral membrane components to internal organelles and coordinate consecutive stages of intracellular transport [9,10]. There are over 70 rabs in mammalian cells and many of these are associated with a single organelle or membrane trafficking step [9,10]. The active or GTP-bound form of a specific rab recruits specific effector proteins (such as those involved in tethering, fusion or targeting), to transduce the signal of the rab GTPase in the transport mechanism of a specific endosome [10]. For example, rab5 and its effector EEA1 regulate clathrin-coated vesicles transport from the plasma membrane to early endosomes [11]. Three major rab proteins, namely rab5, rab4 and rab11 are differentially associated with early endosomes [9]. Endosomes that contain primarily rab5 apparently mediate rapid trafficking “*fast route*” from early endosomes directly back to the membrane. Those that contain both rab5 and rab4 correspond to early/sorting endosomes [9,10]. Endosomes that harbor rab4 and particularly rab11 are termed perinuclear-recycling endosomes, which traffic cargo back to the membrane at a rate slower than the “*fast route*” [12]. Early/sorting endosomes contain tubular and vesicular domains [12–14]. Cargo destined for recycling is segregated to tubular domains, which pinch off to form recycling vesicular endosomes [13,14]. Endosomal structures that remain after vesicular pinching from early/sorting endosomes mature into late endosomes/lysosomes that express rab7 and rab9 [9]. Rab7 is involved in the delivery of early endosomal constituents to late endosomes and in fusing late endosomes with one another [15]. Rab9 is involved in the process of collecting Mannose-6P receptors into vesicles bound for the golgi complex [10].

Partial mapping of intracellular trafficking of a number of GPCR revealed that trafficking between the various endosomal compartments was dependent on preset itineraries. Where by, GPCR that were destined to recycle, trafficked from early/sorting endosomes directly to the membrane “*fast route*” or via a “*slow route*” to the endocytic recycling compartment (ERC), previously referred to as recycling endosomes, prior to their insertion into the membrane [1, 5–8]. For example, the majority of the agonist-internalized β 2-AR and vasopressin type2 receptors (V2R) recycled through the “*fast route*” from sorting endosomes to the membrane, while a minority of these receptors was diverted to the ERC prior to their recycling and reinsertion into the cell membrane [8,16]. For recycling GPCR, there appears to be two routes out of the ERC, a route from the ERC to the membrane and another route from the ERC to the trans golgi network (TGN) and then to the membrane [1]. On the other hand, nonrecycling GPCR traffic from sorting endosomes into late endosomes/lysosomes where they are eventually degraded [17]. Since the internalized WT β 1-AR recycled efficiently while the S312A β 1-AR mutant did not recycle, we compared their intracellular trafficking routes to determine if differential endosomal sorting could account for their divergent trafficking routes.

2. Materials and Methods

2.1. Construction of mammalian expression vectors and shRNA expressing vectors

In order to allow rapid assessment of cell surface expression of the WT β 1-AR or S312A β 1-AR, the N-terminal initiator methionine was replaced by the FLAG sequence DYKDDDDK sequence and these constructs were cloned in the pcDNA 3.0 mammalian expression vector. Each cDNA was transfected into HEK-293 cells and stable expressors were selected with G418. Cell lines expressing equal amounts of β 1-AR ($B_{\max}$ for WT β 1-AR were 584 ± 26 fmoles receptor/mg protein and for the S312A the $B_{\max}$ was 612 ± 30 fmoles/receptor/mg protein). The sequence for each of the 21-bp siRNA constructs were designed using Invitrogen Block-iT RNA designer™, <https://rnaidesigner.invitrogen.com/rnaiexpress/design.do>. These sequences are listed in supplemental Table 1. The algorithm was also used to design double-stranded cDNAs that were cloned into BLOCK-iT™ Pol II miR RNAi Expression Vector with EmGFP. Upon transient transfection into cells, expression of the shRNA is monitored by GFP fluorescence ($\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 520$ nm). To determine the effect of transient expression of 5 μ g of each shRNA/60-mm plate on its target, we probed total RNA by RT-PCR [18] or protein by Western blotting. For the RT-PCR procedure, first-strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit from Roche Diagnostics, (Indianapolis, IN, USA) using 62 ng of RNA per assay. The RT-PCR primers were designed using a web-based design center (www.universalprobelibrary.com) and their sequences are listed in supplemental Table 1. The mRNA level for each protein was quantified using the Universal Probe Library of short hydrolysis locked nucleic acid probes in combination with the primers. The quantification of mRNA was accomplished using the Roche Lightcycler 480 Real-time PCR system and software.

2.2. Primary and secondary antibodies

Antibodies to the rab-GTPase proteins were rabbit or goat primary antibodies purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The following antibodies were used: Anti-rab 5a (sc-309), anti-rab4a (sc-312), anti-rab9 (sc-6562) anti-lamp1 (sc 5570), anti-EEA1 (sc-33585), anti-rab11 (sc-9020), anti-rab11a (sc 26590), anti-rab11b (sc26591). FITC-conjugated anti-human Tfr (CD-71) was from Accurate Chemical and Scientific Corporation (Westbury, NY). Anti-FLAG antibodies were from Sigma (St. Louis, MO). FITC- conjugated or Texas Red-conjugated anti-rabbit, anti-mouse or anti-goat antibodies were from Vector laboratories (Burlingame, CA). Cy3-conjugated goat anti-rabbit IgG was from Millipore Corp. (Billerica, MA).

2.3. Acid strip recycling protocol

In those experiments involving shRNAs, HEK-293 cells were transiently transfected with 1 μ g of Flag-tagged WT β 1-AR and with 5 μ g of plasmid harboring the scrambled or active shRNA-EGFP cDNA. One day after transfection, the cells were trypsinized and plated onto poly-L-lysine coated cover slips in 35-mm plates. After 2 days, the cells were serum-starved at 37°C for 1 h in DMEM supplemented with 25 mM HEPES, pH 7.4, followed by labeling the receptors with Cy3-conjugated, anti-FLAG M2 IgG (10 μ g/ml) for 1 h at 37 °C. Cells were treated with 10 μ M isoprenaline for 30 min at 37 °C to promote agonist-mediated receptor internalization. Then the cells were chilled in 4 °C Tris-buffered saline to stop endocytosis and exposed to 0.2 M NaCl, 0.5 M acetic acid (pH 3.5) for 4 min on ice to remove antibody bound to extracellular β 1-AR [4,19]. Cultures were quickly rinsed in warm Dulbecco's modified Eagles medium (DMEM) supplemented with serum and HEPES and 100 μ g/ml of the lysosomal inhibitor leupeptin, then incubated with 100 μ M of the β -antagonist alprenolol (ALP) at 37 C for 10, 20, 30 or 60 min to establish the recycle time. After each time period, the cover slips were rinsed and fixed in 4% paraformaldehyde with 4% sucrose in PBS (pH 7.4) for 10 min at room temperature.

2.4. Confocal microscopy and analysis of immunocytochemical data

Analyses were performed blind to the stimulation history of the culture. Microscope fields that had 1–3 cells displaying generally healthy morphology and manifested GFP fluorescence were visualized. Six to ten fields were imaged per culture and $n = 10$ cultures were processed per condition. Confocal fluorescence microscopy was performed using a Zeiss Axiovert LSM 510 [100 × 1.4 DIC oil immersion objective]. FITC was excited with the 488-nm argon laser and imaged through the 520-nm long-pass emission filter and Texas red was excited with at 543 nm and imaged through the 560 LP filter. Thresholds were set by visual inspection and kept constant for each condition. Z-stacks of images were exported as TIFF files and individual sections were analyzed with Zeiss LSM 510 and NIH Image 1.6 software as previously described [3].

To determine the distribution of receptors between the membranous and intracellular compartments, a circular boundary was drawn around the inner circumference of all acid/stripped GFP-positive cells to define a 300-nm wide membrane delimited area. Fluorescence intensity measurements were calculated in the areas outside and inside the boundary to estimate the membranous versus internal pixels. These measurements were repeated in slides with similar threshold setting in which the radii for each boundary did not differ by $\pm 15\%$. Pixel intensities in the intracellular boundary (internalized $\beta 1$ -AR) of isoprenaline-treated cells were set arbitrarily as 100% and pixel intensities in the intracellular boundaries of alprenolol-treated cells were calculated as percent of this initial value. The data are presented as the mean $\pm$ S. E. of three independent experiments each involving between 10–20 cells.

2.5. Dual confocal microscopy

HEK-293 cells stably expressing the FLAG-tagged WT or S312A $\beta 1$ -AR were cultured in DMEM +10% FBS supplemented with 100 $\mu\text{g}/\text{ml}$ of leupeptin. Cells on cover slips were exposed to 10 μM isoprenaline for 5 min. Then, the medium was aspirated and replaced with culture medium containing 100 μM of the β -antagonist alprenolol. After 0, 5, 15 and 30 min from the addition of alprenolol (*i.e.* 5, 10, 20 and 25 min from the addition of isoprenaline), the cover slips were fixed with 4% paraformaldehyde and then permeabilized with 1% triton X-100. The cells were stained with anti-FLAG IgG and with the primary polyclonal antibody to each endosomal protein under study. Then FITC-, or Cy3/Texas red-conjugated anti-primary (rabbit, mouse or goat) antibody was added for 1 h to label the primary antibody. The cells were visualized by dual confocal microscopy (GFP, $\lambda_{\text{ex}} = 488$ nm, $\lambda_{\text{em}} = 505$ –530 BP, Cy3/Texas-red, $\lambda_{\text{ex}} = 543$ nm, $\lambda_{\text{em}} = 560$ LP) using LSM-510 multitracking configuration as described [3,4].

Quantitative immunofluorescence was performed using a 100X objective [1.4 N.A.] on Zeiss LSM510 microscope equipped with a Zeiss Axiovision cooled CCD camera. Quantification of signal overlap was determined using Pearson Correlation Coefficient (Zeiss software) on thresholded images and calculating (number of double positive pixels)/(total number of $\beta 1$ -AR-positive pixels) for each endosome marker. Thresholds were set by visual inspection and kept constant for each condition. Data were analyzed from four to six cells from three separate coverslips ($n = 16$ images) for each double-labeling experiment. Results represent mean $\pm$ S.E. of percentile overlap between the $\beta 1$ -AR and the rab protein versus time. To determine the statistical significance of the colocalization between the WT $\beta 1$ -AR *versus* the S312A with each rab protein at a given time point, the % colocalization for the WT $\beta 1$ -AR-rab ($n=16$) *versus* the % colocalization of the corresponding S312A $\beta 1$ -AR-rab ($n=16$) were analyzed by the PrismTM program (Graphpad Corp.). Statistical analysis of % colocalization of WT $\beta 1$ -AR-rab vs. S312A-rab at each time point was determined by the non-parametric *t* test subroutine of the Mann-Whitney algorithm. *P* values <0.05 were considered significantly different and

denoted with an asterisk (*), while $p < 0.01$ or $p < 0.001$ were denoted with a double (**) or triple asterisk (***)

2.6. Assay of β_1 -AR degradation by Biotinylation

Cells expressing the WT β_1 -AR or the S312A β_1 -AR were washed twice with ice-cold PBS and then biotinylated with 1.5 mg/ml sulfo-NHS-SS-biotin on ice for 20 min, and the excess biotin was quenched with 50 mM glycine [2]. Biotinylated cells were exposed to isoprenaline for 0, 2, 4 and 6 h and then lysed by sonication in lysis buffer composed of (50 mM Tris pH 7.4, 2 mM EDTA, 2 mM EGTA), plus phosphatase inhibitors (50 mM NaF, 1 mM Na_3VO_4) and protease inhibitors (10 μM aprotinin, 0.1 mM phenylmethyl sulfonylfluoride, 0.1 mg/ml benzamide, plus 10 $\mu\text{g}/\text{mL}$ each of chymostatin, pepstatin, antipain, and leupeptin), followed by centrifugation at $100,000g_{\text{av}}$ for 20 min. The membrane pellet was extracted in lysis buffer containing 0.2% SDS and 1% triton X-100 followed by centrifugation at $100,000g_{\text{av}}$ for 20 min. The supernatants were collected and assayed for protein. Then equal amounts of protein were incubated by inversion with 50 μL of BSA-blocked Ultralink-neutravidin beads (Pierce) for 2 h at 4 °C. The beads were washed in lysis buffer with detergents and the biotinylated proteins were eluted in Laemmli buffer and subjected to SDS-PAGE followed by electro-transfer to nitrocellulose filters [2]. The filters were probed with anti-FLAG M2 monoclonal antibody and visualized with HRP-labeled goat anti-mouse IgG (Upstate Biotechnology).

3. RESULTS

3.1 Internalized WT- and S312A- β_1 -AR traffic to early endosomes

Our experimental approach for mapping the trafficking of the WT β_1 -AR *versus* the S312A β_1 -AR mutant involved dual localization confocal microscopy between internal β -AR and endosome-specific rab proteins. To ascertain that anti-rab antibodies were specific for a given rab protein, we conducted co-localization studies with FITC- or Cy3-labeled anti-rab antibodies that were directed to different domains in each rab protein (Fig. 1S, panel A). These experiments confirmed that the anti-rab antibodies used in this study were highly specific. Next we determined the distribution of β -AR and rab proteins in unstimulated HEK-293 cells (Fig. 1S, panel B). The data indicated that β_1 -AR were localized in the membrane of control cells while rab proteins were intracellularly distributed, with little overlap (<20%) between them.

Trafficking experiments were conducted in HEK-293 cells stably expressing either the WT β_1 -AR or its S312A mutant. Trafficking was initiated by exposing cells to isoprenaline for 5 min, a time point at which the β_1 -AR would undergo significant internalization, but no substantial recycling [2]. After 5 min, the internalization of the β_1 -AR, but not its trafficking was blocked with the β -antagonist alprenolol. After 5, 10, 20 and 35 min from the addition of isoprenaline, the cells were fixed, permeabilized and incubated with primary antibodies to the FLAG epitope (to visualize β_1 -AR) and to the rab protein under study. Then the cells were incubated with FITC- (green) or Texas red/Cy3- (red) labeled secondary antibodies. At each time point, two-color confocal images between the β_1 -AR and the rab protein were acquired. The percentile co-localization between the two fluorescent dyes was calculated using the multitracking configuration software of the microscope.

Cargos including GPCR that are internalized either by clathrin-coated pits or via caveolae are delivered initially to early sorting endosomes, which express rab5a and its binding partner EEA1 [15,20–22]. In untreated cells, the percentile overlap between the β_1 -AR and the early endosomal marker rab5a was $\sim 10 \pm 2\%$ (Fig. 1B). In response to a brief 5 min application of isoprenaline, WT β_1 -AR and S312A β_1 -AR were internalized from the membrane to intracellular compartments that displayed punctate patterns through out the cytoplasm (Fig. 1A images a, e, i and m). These β_1 -AR positive structures displayed extensive colocalization

markers for early sorting endosomes, such as FITC-labeled rab5a ($74 \pm 6\%$ of internal WT $\beta 1$ -AR and $62 \pm 6\%$ of internal S312A $\beta 1$ -AR) and FITC-labeled EEA1 (72 ± 7 of internal WT $\beta 1$ -AR and 61 ± 7 of internal S312A). By 10 min after the addition of isoprenaline, colocalization between the WT $\beta 1$ -AR and rab5a decreased to $\sim 40\%$, while colocalization between the S312A $\beta 1$ -AR and rab5a was close to that in untreated cells. The percentile colocalization between the WT $\beta 1$ -AR and rab5a/EEA1 at 10 min vs. the percentile colocalization of S312A and rab5a/EEA1 at 10 min were significantly different ($p < 0.05$), indicating that by 10 min the S312A $\beta 1$ -AR had exited the rab5a-containing compartment (Fig 1 *images b–d, h–f, j–l* and *n–p*). We observed in some cells, particularly in latter time frames (>20 min after the addition of isoprenaline) that the WT $\beta 1$ -AR (red pixels) recycled back into the membrane compartment (Fig. 1A, *images c, d* and Fig. 1C *image l*). On the other hand and in the same time frames, S312A $\beta 1$ -AR was retained intracellularly reinforcing the notion that the S312A mutation inhibited the recycling of the $\beta 1$ -AR (Fig. 1A and C). In supplemental Fig. 2S, we are providing single color and dual color images of the data in Fig. 1A and 1C to illustrate how the raw data between FITC- and Cy3/Texas red-labeled proteins was used to estimate the percentile overlap between them.

3.2. Role of rab4 in trafficking the WT $\beta 1$ -AR versus the S312A $\beta 1$ -AR mutant

Next, we determined the distribution of $\beta 1$ -AR with rab4 GTPase, which mediates the transfer of cargo from early sorting endosomes into recycling endosomes [9]. Rab4a colocalized by $63 \pm 8\%$ with the WT $\beta 1$ -AR within 5 after its internalization and by 10 min their colocalization was $\sim 51 \pm 6\%$ (Fig. 2A, *image a, b*). After 10 min, the WT $\beta 1$ -AR was no longer colocalized with rab4a (Fig. 2A, *images c–d* and panel B). Therefore, the WT $\beta 1$ -AR trafficked through rab5a and rab4a-containing early endosomes. On the other hand, the association between rab4a and S312A $\beta 1$ -AR was $\sim 22 \pm 4\%$ (Fig. 2A, *image e*) suggesting that S312A $\beta 1$ -AR did not traffic through rab4a-containing endosomes (Fig. 2A *images f–h*, and panel B). The colocalization between the WT $\beta 1$ -AR and rab4a at 5 and 10 min vs. the colocalization of S312A and rab4a at 5 and 10 min were significantly different ($p < 0.01$), indicating that S312A $\beta 1$ -AR did not colocalize with rab4a.

To test the hypothesis that the trafficking routes of the WT $\beta 1$ -AR and the S312 $\beta 1$ -AR were differentially dependent on rab4, we compared the trafficking routes of the WT $\beta 1$ -AR and S312A $\beta 1$ -AR with that of transferrin bound to its receptor (Tfr). The Tfr pathway is a canonical pathway for constitutive internalization and recycling [15]. In this pathway, the Tfr is first internalized into rab5/EEA1 positive compartments and then traffics through rab4-positive structures prior to its reinsertion into the membrane [15,22]. Within 5 min after the addition of isoprenaline, the association between the internalized WT $\beta 1$ -AR and Tfr was relatively high at 70% (Fig. 2C *images i–j* and panel 2D). Significant colocalization between the Tfr and the WT $\beta 1$ -AR was maintained for an additional 10 min, but after 20 min their colocalization was reduced to $<20\%$ (Fig. 2C *images k–l* and panel 2D). Therefore, as seen previously with rab4a, the trafficking routes of the WT $\beta 1$ -AR diverged significantly from the Tfr after their exit from rab5a/rab4-expressing early endosomes. Colocalization between the Tfr and the S312A $\beta 1$ -AR at 5 or 10 min after adding isoprenaline was $\sim 26\%$ (Fig 2C *images m, n*) and was quickly reduced to basal levels ($<20\%$) in latter time frames (Fig. 2D). Colocalization between the WT $\beta 1$ -AR and Tfr at 5 and 10 min vs. the colocalization of S312A and Tfr at 5 and 10 min were significantly different ($p < 0.01$), indicating that S312A $\beta 1$ -AR did not colocalize with Tfr.

3.3. S312A $\beta 1$ -AR traffics from early endosomes to late endosomes/lysosomes

Since the S312A $\beta 1$ -AR did not recycle and was retained internally, we determined whether it was shuttled to late endosomes after its exit from rab5a/EEA1-containing early endosomes. At first, we compared its colocalization with rab9, which localizes primarily in late endosomes and to a smaller extent in the TGN [10]. As expected, in early time frames (5–10 min after the

addition of isoprenaline) the colocalization between S312A and rab9 was low. Then after 20–30 min from the addition of isoprenaline, the S312A β 1-AR trafficked by more than 50% to rab9-positive endosomes (Fig. 3A, *images e–h* and panel 3B). On the other hand, the colocalization between the WT β 1-AR and rab9 was consistently low in early or late trafficking time frames (Fig. 3A *images a–d* and panel 3B). Next, we determined the colocalization of S312A with lamp1, which is a *bona fide* marker for late endosomes/lysosomes [9]. After 10 min from its internalization, the colocalization between S312A and lamp1 was low ($\sim 25 \pm 4\%$), but rapidly increased to $60 \pm 5\%$ in later time frames, indicating that the S312A β 1-AR trafficked into late endosomes/lysosomes (Fig. 3 *m–p* and 3D). The colocalization between the WT β 1-AR and lamp1 was consistently low in early or late trafficking time frames (Fig. 3A *images i–l* and panel 3B). Colocalization between the WT β 1-AR and rab9/lamp1 vs. the colocalization of S312A and rab9/lamp1 were significantly different at early (5, 10 min) time frames ($p < 0.05$ – 0.01). After 20–35 min these differences increased ($p < 0.001$), indicating that the WT β 1-AR did not colocalize with either rab9 or lamp1 in late endosomes/lysosomes during its trafficking.

3.4. Agonist-internalized S312A β 1-AR are degraded in lysosomes

Since the WT β 1-AR and the S312A were differentially associated with lysosomal markers, we compared the rates of degradation of the WT and the S312A β 1-AR mutant in HEK-293 cells that were surface biotinylated and then exposed to isoprenaline for 2–6 h (Fig. 4A). Cellular levels of the WT β 1-AR in cells that were continuously exposed to isoprenaline for 6 h were relatively unchanged, indicating that the internalization and recycling pathways of the WT β 1-AR were highly efficient in maintaining cellular complements of WT β 1-AR (Fig. 4B). However, after 6 h from continuous exposure to isoprenaline, the nonrecycling S312A β 1-AR were degraded by more than 80%, with an estimated $t_{0.5}$ of degradation of ~ 2 h (Fig. 4A and 4B). Comparison between the levels of WT β 1-AR vs. those of the S312A in HEK-293 cells after 2–6 h from the addition of isoprenaline indicated that the levels of WT β 1-AR were significantly greater than those of the S312A β 1-AR ($p < 0.001$).

3.5. Involvement of the rab11-GTPase in sorting and recycling of WT β 1-AR

Thus far we have determined that in early time frames (5–10 min after internalization) the trafficking WT β 1-AR was associated with rab5a/EEA1 and rab4-containing early sorting endosomes. Subsequently, the association between the WT β 1-AR and these endosomal markers declined, suggesting that the WT β 1-AR exited from early sorting endosomes into another compartment. To better define this compartment, we determined the colocalization between the WT β 1-AR and several rab proteins in latter time frames (10 min or later after the addition of isoprenaline). In unstimulated cells the association between the WT β 1-AR and rab11 was $\sim 12\%$ (Suppl. Fig. 1). The data in Fig. 5 indicate that by 20–30 min after the addition of isoprenaline, the association between the WT β 1-AR and rab11 increased to $67 \pm 8\%$ (Fig. 5A compare *images c–d* with *a–b*). Since the rab11 GTPase is involved in sorting and recycling of cargo [9], these data indicate that the internalized WT β 1-AR was sorted from rab5a- and rab4-positive endosomes into rab11-containing recycling endosomes (Fig. 5B). Unlike the WT β 1-AR, the colocalization between S312A β 1-AR and rab11 did not exceed 20% in any of time points that were measured (Fig. 5A *images e–h* and 5B). Colocalization between the WT β 1-AR and rab11 vs. the colocalization of S312A and rab11 were significantly different ($p < 0.001$) in all the time points that were measured, indicating that the S312A β 1-AR did not colocalize with rab11 in recycling endosomes.

3.6. rab11a mediates the trafficking of the WT β 1-AR

To characterize the role of rab11 in regulating the recycling of the WT β 1-AR we determined the role of WT rab11 and its functionally altered mutants on the kinetics of β 1-AR recycling

by confocal microscopy (Fig. 6). We cotransfected cells with plasmids harboring the WT β_1 -AR with one of three rab11 cDNAs that express WT rab11, dominant negative rab11 or constitutively active rab11 and then determined the effect of each rab11 construct on internalization and recycling of the WT β_1 -AR [23]. Coexpression of WT β_1 -AR and WT rab11 did not significantly affect the internalization of the WT β_1 -AR and these receptors were distributed into punctate structures that were observed earlier in empty vector expressing HEK-293 cells (Fig. 6A, *images b and h*). WT rab11 did not accelerate the recycling of the agonist-internalized WT β_1 -AR because the $t_{0.5}$ for recycling of the WT β_1 -AR in control cells was 24 ± 5 min and in WT rab11-expressing cells the $t_{0.5}$ was 21 ± 4 min [$n=4$, $p>0.05$] (compare *images b-f* to *h-l* and panel 6B). The dominant negative mutant of rab11 (S25N) effectively inhibited the recycling of the WT β_1 -AR, where by these receptors were concentrated in a very large endosomal compartment in close proximity to the nucleus (Fig. 6A, *images n-r*). Boundary measurement of cells after 10 to 60 min from the removal of isoprenaline indicated that in cells expressing S25N rab11 almost all the receptors were retained inside the 300-nm cytoplasmic boundary (Fig. 6A *images n-r* and panel 6B). In these time frames, the distribution of WT β_1 -AR in S25N expressing cells was significantly different from their distribution in control cells ($p < 0.001$). The constitutively active (Q70L) rab11 did not affect the internalization of the WT β_1 -AR, but it accelerated the recycling of the WT β_1 -AR to a $t_{0.5}$ of 15 ± 3 min (Fig. 6A *images t-x* and panel 6B), which was significantly shorter than the $t_{0.5}$ for the recycling of the WT β_1 -AR ($n=4$, $p<0.02$).

To characterize the involvement of each isoform of rab11 on recycling of the WT β_1 -AR, we generated short hairpin RNA (shRNA) against the two isoforms of rab11 (Fig. 6C). We determined that the rab11a shRNA selectively down-regulated the rab11a protein by >85% and that the rab11b shRNA down regulated rab11 b by ~70% (Fig. 6D). Confocal microscopy revealed that the shRNA to rab11a significantly inhibited the recycling of the WT β_1 -AR (compare *image f'* in Fig. 6C with *image c'*), while the shRNA to rab11b did not affect the rate or extent of recycling of the WT β_1 -AR (compare *image i'* in Fig. 6C with *image c'*). Thus, rab11a was solely involved in trafficking of the WT β_1 -AR in HEK-293 cells.

3.7. Rab11-interacting proteins regulate rab11-mediated recycling of the WT β_1 -AR

One of the molecular mechanisms by which rab11 performs its regulatory role in intracellular trafficking involves its binding to rab11 interacting proteins. These include myosin Vb [24], rab11-binding protein/Rabphilin [23,24,25], and a group of rab11-family interacting proteins [rab11-FIPs] such as rab11-FIP1 (also called rab-coupling protein (RCP), rab11-FIP2, rab11-FIP3, rab11-FIP4 and rab11-FIP5 (also called Rab11-interacting protein (RIP)), rab6 interacting protein (rab6-ipi) and the neuronal protein protrudin [26,27,28,29,30]. Because β_1 -AR recycling is essential for resensitizing and restoring β_1 -AR-mediated signaling, we determined whether knockdown of each rab11 interacting protein would affect the trafficking of the WT β_1 -AR in HEK-293 cells.

Myosin Vb binds to rab11 and acts a molecular motor to drive vesicular transport on actin tracks. Myosin Vb is specifically recruited to rab11-recycling endosomes because the c-terminal domain of myosin Vb interacts specifically with membrane bound (i.e. GTP-bound) rab11 [24,31]. ShRNA-mediated knockdown of myosin Vb did not alter the kinetics or extent of β_1 -AR internalization in response to isoprenaline (Fig. 7A *image e*). However, the shRNA to myosin Vb inhibited the recycling of the agonist-internalized β_1 -AR and these receptors were clustered in the perinuclear area of the cell (Fig. 7f). In addition to binding rab11a, myosin Vb also binds to the rab11-interacting protein FIP2 and the triple complex consisting of myosin Vb, Rab11a and FIP2 is required for efficient recycling of Niemann-Pick C1 Like 1 Protein NPC111 in the liver [32]. Knockdown of FIP2 had no effect on the internalization of the WT β_1 -AR (Fig. 7, *image h*), but inhibited the recycling of the internalized β_1 -AR (Fig. 7, *image*

i). The distribution of internal $\beta 1$ -AR in cells expressing FIP2 shRNA was similar to their distribution in myosin Vb expressing cells, in that they were clustered in the perinuclear area (Fig. 7, compare image 7f to 7i). We extended the duration of the recycling experiments involving FIP2 and myosin Vb to 4 h and were not able to observe significant recycling. Therefore, knockdown of these proteins did not simply slow down the recycling kinetics, but rather inhibited the recycling of the WT $\beta 1$ -AR.

Another rab11 interacting protein is the rab11-binding protein (rab11BP) or rabphilin-11, which interacts exclusively with the GTP-bound form of rab11a [23,25]. Rabphilin-11 is localized on vesicles in perinuclear regions and on microtubules oriented toward the plasma membrane [33]. Knockdown of rab11BP did not alter the internalization parameters of the WT $\beta 1$ -AR (Fig. 7k), but markedly reduced the recycling of these receptors (Fig. 7l). $\beta 1$ -AR in these cells were confined in punctate vesicles that were distributed throughout the cell, suggesting that knockdown of the rab11-BP affected vesicular transport rather than vesicular fusion (Fig. 7, image l). RIP6ipi is another rab11 interacting protein that links the functions of rab6 and rab11 [34]. Knockdown of rab6ipi did not affect either the internalization or recycling of the WT $\beta 1$ -AR (Fig. 7, images n-o).

In addition to FIP2, there are four other rab11-FIP proteins, which can be divided into two main classes [27,35]. Class I FIPs (FIP1/RCP, FIP5/Rip11, and FIP2) contain a C2 domain at the N-terminus and interact with rab11 GTPase in a GTP-dependent manner [27,35]. Class II FIPs, such as FIP3 and FIP4, contain two EF hands and in addition to interacting with rab11 they have been shown to interact with Arf GTPases [36,37]. Functionally, rab11-FIP3 and FIP4 are ERC proteins that are involved in delivering cargo such as the Tfr from the ERC to sites of membrane insertion [38]. Knockdown of FIP1/RCP or FIP5/Rip11 had no effect on the extent of internalization of the WT $\beta 1$ -AR (Fig. 8 d-e and m-n), or on the recycling kinetics of the WT $\beta 1$ -AR (Fig. 8 images f and o). Knockdown of class II FIPs, FIP3 or FIP4 had no effect on the internalization of the WT $\beta 1$ -AR in response to isoprenaline (Fig. 8A, images g-h and j-k). In cells in which FIP3 was knocked down, we did not observe significant recycling of the WT $\beta 1$ -AR and internal receptors were confined in punctate vesicles that were distributed throughout the cell (Fig. 8A, image i). Under the same experimental conditions, knockdown of FIP4 had no effect on the recycling of the WT $\beta 1$ -AR (Fig. 8A, image l).

3.8. Myosin VI regulates the internalization of the WT $\beta 1$ -AR in response agonists

Finally, we investigated the effect of myosin VI on trafficking of the WT $\beta 1$ -AR (Fig. 8B). Myosin VI is an unconventional myosin that travels toward the minus end of F-actin, which is oriented away from the plasma membrane [39]. In this regard, myosin VI would more likely be involved in endocytosis because it colocalized with clathrin-coated pit forming proteins such as clathrin and the clathrin adapter protein AP-2 [40,41]. ShRNA-mediated knockdown of myosin VI, inhibited the internalization of the WT $\beta 1$ -AR by >80% (Fig. 8B, compare image p to q). To conclusively demonstrate that knockdown of myosin VI inhibited the internalization of WT $\beta 1$ -AR, we exposed cells pretreated with isoprenaline (Fig. 8B, image q) to a NaCl/ acetic acid “acid wash” solution to strip the Cy3-anti-Flag IgG from $\beta 1$ -AR that were retained on the cell surface [19]. Images of cells before and after “acid wash” show that this maneuver stripped >80% of the Cy3 pixels, indicating that the majority of $\beta 1$ -AR in myosin VI-knockdown cells were not internalized in response to isoprenaline (compare image r in Fig. 8B to image q).

Discussion

We have shown that the serine 312 to alanine point mutant of the human $\beta 1$ -AR is internalized normally, but unlike the WT $\beta 1$ -AR, this $\beta 1$ -AR mutant did not recycle or resensitize the $\beta 1$ -AR signaling pathway [2]. Since this mutation interferes with PKA-mediated phosphorylation

of the $\beta 1$ -AR, it is reminiscent of the Ser256 mutation in aquaporin-2 that also inhibited the translocation of aquaporin from intracellular stores into the membrane [42]. These reports highlight the critical role of PKA in the setting the trafficking itinerary of proteins involved in signaling and in transfer of other molecules across cell membranes. In this report we have shown that this point mutation interfered in each trafficking step of the $\beta 1$ -AR. Our studies indicate that even though both the WT $\beta 1$ -AR and the S312A $\beta 1$ -AR were endocytosed into rab5a/EEA1 containing early endosomes, these endosomes were heterogeneous and each receptor was internalized into a distinct population. These observations were based on two crucial findings. First, we determined that the S312A $\beta 1$ -AR was internalized into early endosomes that contained rab5a/EEA1 proteins only, while the WT $\beta 1$ -AR was internalized into endosomes that contained rab5a/EEA1 and rab4 proteins (Fig. 1 and 2). Second, trafficking of the WT $\beta 1$ -AR *versus* the S312A out of this compartment displayed different exit kinetics. Whereby, colocalization between S312A and rab5a returned to control value within 10 min after the addition of isoprenaline, while the exit kinetics of the WT $\beta 1$ -AR were much slower (Fig. 1). These two pieces of data strongly support the hypothesis that early on, the WT $\beta 1$ -AR and the S312A $\beta 1$ -AR trafficked into different populations of early endosomes. This notion was further strengthened by the Tfr trafficking data, which also indicated that the Tfr trafficked into a different endosomal compartment than the S312A $\beta 1$ -AR. The rapid exit of S312A $\beta 1$ -AR out of rab5a/EEA1-containing endosomal compartment was not surprising because these endosomes mediate rapid exit trafficking kinetics [9]. Temporal trafficking kinetics of the WT $\beta 1$ -AR and Tfr clearly indicated that these receptors were internalized initially into rab5a/EEA1 and rab4-positive early sorting endosomes [9,10]. The role of rab4 in this phenomenon is crucial because rab4-positive tubules frequently grow out and detach from rab5-positive cores [43]. These endosomes display slow trafficking kinetics, which is what we observed with the WT $\beta 1$ -AR (Fig. 1–2). Our data also highlighted that the trafficking routes of the WT $\beta 1$ -AR and Tfr were divergent. In that the association between the Tfr and the WT $\beta 1$ -AR was reduced to almost control levels by 20 min after the addition of isoprenaline. These results are in agreement with studies that have shown divergence in the recycling pathways of the Tfr and those of the $\beta 2$ -AR and N-methyl-D-aspartate (AMPA) receptors particularly in later time frames [44,45,46].

The exit of S312A out of early endosomes occurred within 5–10 min from its internalization and its colocalization with rab9 and lamp1 in late endosomes occurred after 20 min from its internalization. Based upon the gap of 5–10 min in the trafficking timeline of the S312A $\beta 1$ -AR, it is conceivable that an intermediate could have been involved between the delocalization of S312A $\beta 1$ -AR from rab5a to its colocalization with rab9/lamp1 (Fig. 1 and 3). The temporal relationship between these processes might be related to the conversion of rab5 in early endosomes to rab7 during early to late endosome transport. This process is regulated by the homotypic fusion and vacuole protein sorting complex and other proteins such as the N-ethylmaleimide sensitive fusion protein among others [43]. In this report we explored the roles of rab9 and lamp1 in exit of the S312A $\beta 1$ -AR from early endosomes because we wanted to determine if the TGN was involved in this process. Rab9 localizes primarily in late endosomes and to a smaller extent in the TGN [47]. To determine whether the S312A $\beta 1$ -AR mutant trafficked via the TGN *en route* from sorting endosomes to late endosomes/lysosomes we compared temporal localization of S312A with rab9 and with the *bona fide* late endosomal marker lamp1. We determined that temporal association between lamp1 and rab9 with the S312A $\beta 1$ -AR was very comparable indicating that the S312A $\beta 1$ -AR did not traffic through the TGN (Fig. 3). Thus, ultrastructural and biochemical data in figure 4 are in agreement that colocalization of the S312A $\beta 1$ -AR protein with lysosomal markers resulted in significant degradation of this receptor.

In addition to S312A we also determined temporal relationships between the WT $\beta 1$ -AR and trafficking rab proteins. The WT $\beta 1$ -AR consistently accumulated in rab5/EEA1 and rab4-

positive endosomes, which eventually lost rab5 and rab4. Remarkably, the rab11 signal increased in parallel resulting in conversion of the endosomal membrane from rab5 positive/rab11 negative to rab5 negative/rab11 positive within 15–20 min after the internalization of the WT β 1-AR (Fig. 5). The dependence on rab11 in the recycling of the WT β 1-AR highlights a major difference in the recycling itinerary of the β 1-AR *versus* that of the β 2-AR. In the case of β 2-AR, most of these receptors recycled directly from early endosomes to the cell surface via the fast recycling pathway and only a small fraction of β 2-AR recycled through the slow pathway via the rab11-recycling compartment [16,44,45]. Thus, it appears that rab11 plays a small role in recycling of the β 2-AR, while it plays a dominant role in recycling of the WT β 1-AR. A differential role for rab11 in trafficking of two subtypes of a given GPCR was observed in the trafficking of the two subtypes of vasopressin receptors [8,47]. Agonist internalized vasopressin type 1 receptor (V1R) recycled rapidly and efficiently while the internalized V2R did not recycle [48]. The agonist-internalized V1R recycled via the fast recycling pathway in a rab11-independent manner. Surprisingly, the V2R trafficked from early endosomes to a rab11-containing pericentriolar compartment. Thereafter, V2R trafficked from pericentrioles to late endosomes/lysosomes where they were eventually degraded [48]. In this regard, endosomes enriched in rab11 were not involved in recycling of the V2R rather they served as a conduit to its degradation. Thus, rab11-containing pericentriolar endosomes either required additional trafficking information for recycling or these endosomes were heterogeneous containing different subsets of recycling and nonrecycling vesicles. The latter seems a more likely scenario because the Tfr, β 1-AR, CFTR and many other proteins recycled through the rab11-containing pericentriolar compartment [23,49]. The V2R however, was colocalized with rab11, but since it did not recycle, it would indicate that V2R might have accessed a subset of nonrecycling pericentriolar endosomes. The nonrecycling S312A β 1-AR mutant did not colocalize with rab11, perhaps because it lacked the necessary targeting information to enter this compartment and trafficked instead from early endosomes to late endosomes/lysosomes.

The dependence of trafficking of the WT β 1-AR on rab11a led us to explore the role of rab11-interacting proteins in this phenomenon. Among the various rab11-interacting partners, myosin Vb has been implicated in recycling of other GPCR. In addition to the β 1-AR, myosin Vb influenced the endocytic shuttling and sorting of the β 2-AR [4], the M4 muscarinic [31], the chemokine CXCR2 [50], the aquaporin-2 receptor [51] and other proteins such as the Tfr [23], CFTR [52] and AMPA receptors [53]. We expanded the observation on the role of myosin Vb on recycling by implicating its rab11-binding partner FIP2. In neurons, activation of NMDA receptors increases calcium influx as well as the association of myosin Vb with recycling endosomes via the recycling endosomal adaptor FIP2 [54,55]. Interference with the formation of the myosin Vb and FIP2 complexes abolished long-term potentiation-induced neuronal plasticity [54]. These data provide a blueprint for future research concerning the role of these trafficking proteins in recycling-mediated resensitization of GPCR signaling [1].

In addition to implicating myosin Vb we implicated myosin VI in trafficking of the WT β 1-AR. This protein has not been implicated in GPCR trafficking, but it potently inhibited the internalization of the WT β 1-AR. These data are important because the tail of myosin VI binds cargo linker proteins such as SAP97 that differentially localize myosin VI to distinct populations of clathrin-coated pits [56]. We have determined that SAP97 also binds to the type 1 PDZ “ESKV” sequence at the carboxy-terminus of the human β 1-AR to assemble a receptosome composed of the β 1-AR, SAP97 and AKAP79/PKA [4]. This complex is involved in agonist- and PKA-mediated phosphorylation of Ser312 of the β 1-AR and therefore, in setting the trafficking itinerary of the β 1-AR. Conceptually, the complex formed at the carboxy-terminus could play a pivotal role in the internalization as well as in the recycling of the β 1-AR. Our data concerning the roles of myosin VI and myosin Vb in the internalization and recycling of the β 1-AR are in agreement with the results of Swiatecka-Urban et al., [52], which

have shown that myosin Vb and myosin VI were involved in recycling and internalization of the CFTR, respectively. These findings underscore the role of actin filaments in the internalization and recycling of the human WT $\beta 1$ -AR. Whereby, myosin VI is apparently involved in facilitating the endocytosis of the $\beta 1$ -AR towards the F minus end of actin while myosin Vb was involved in facilitating the exocytosis of internal $\beta 1$ -AR towards the F plus end of actin filaments.

Our results highlight the interplay between the various rab proteins in temporal regulation of human $\beta 1$ -AR trafficking in HEK-293 cells. This report is the first to show the effect of a single point mutation (S312A) on trafficking of a GPCR. These findings show that the trafficking routes of recycling WT $\beta 1$ -AR and nonrecycling S312A $\beta 1$ -AR diverged shortly after internalization. Where by, the WT $\beta 1$ -AR trafficked in a rab4-dependent manner from rab5a/EEA1 containing early endosomes to rab11-positive recycling endosomes, while the S312A trafficked in a rab4a/rab11-independent manner to late endosomes/lysosomes. These novel findings indicate that trafficking information is imprinted into the GPCR protein shortly after its activation by the agonist. The kinetics of association and dissociation between the WT $\beta 1$ -AR and rab5a/EEA1 or with rab4a/Tfr in HEK-293 cells were quite similar to the trafficking kinetics between these rab proteins and AMPA receptors in neurons, suggesting that this pathway might be a common route for trafficking of a large subset of signaling molecules [46]. In addition we implicated three additional rab11-binding proteins in trafficking of the WT $\beta 1$ -AR. These results should provide a framework for future studies to identify factors that are involved downstream of rab11 (such as SNAP, SNAREs and the exocyst complex proteins) in recycling GPCR into their proper membrane compartment.

This study provides a comprehensive picture of the factors involved in sorting the agonist-internalized GPCR. A better understanding of the basic mechanisms of GPCR trafficking is a priori for elucidating the factors involved in trafficking defects of GPCR that are observed in diseases such as heart failure and others [57].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank David Sabatini NYU Medical Center for providing the rab11 plasmids, the NEI Vision Core [NEI 5P30EY13080-10, D. Johnson, P.I.] and the Confocal microscopy unit at the Molecular Resources Center, UTHSC. This publication was made possible by Grant-in-aid 0855017E from the Greater SE -AHA and HL-085848 from NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the AHA or NIH.

List of abbreviations

$\beta 1$-AR	$\beta 1$ -adrenergic receptors
S312A	serine ³¹² to alanine mutant of $\beta 1$ -AR
WT	wild type
EEA1	Early endosomal antigen-1
GPCR	G protein-coupled receptor(s)
PKA	cyclic AMP-dependent protein kinase
PDZ	PSD-95/DLG/ZO1
ERC	endocytic recycling compartment

V2R	vasopressin type2 receptor
ERC	Endocytic recycling compartment
TGN	trans-golgi network
HEK	human embryonic kidney
ISO	Isoprenaline
Tfr	transferrin receptors
shRNA	short hairpin interfering RNA
ALP	Alprenolol
β2-AR	β2-adrenergic receptor
CFTR	cystic fibrosis transmembrane conductance regulator
FIP	rab11-family interacting proteins
AMPA	N-methyl-D-aspartate
GFP/EGFP	enhanced green fluorescent protein
DMEM	Dulbecco's modified essential medium
FITC	fluorescein isothiocyanate
NHS	N-hydroxysuccinimide
AKAP	A-kinase anchoring proteins

References

1. Hanyaloglu AC, von Zastrow M. *Annu Rev Pharmacol Toxicol* 2008;48:537. [PubMed: 18184106]
2. Gardner LA, Delos Santos NM, Matta SG, Whitt MA, Bahouth SW. *J Biol Chem* 2004;279:21135. [PubMed: 14990580]
3. Gardner LA, Tavalin SA, Goehring A, Scott JD, Bahouth SW. *J Biol Chem* 2006;281:33537. [PubMed: 16940053]
4. Gardner LA, Naren AP, Bahouth SW. *J Biol Chem* 2007;282:5085. [PubMed: 17170109]
5. Millman EE, Zhang H, Zhang H, Godines V, Bean AJ, Knoll BJ, Moore RH. *Traffic* 2008;11:1958. [PubMed: 18785920]
6. Fan GH, Lapierre LA, Goldenring JR, Sai J, Richmond A. *Mol Biol Cell* 2004;15:2456. [PubMed: 15004234]
7. Bremnes T, Paasche JD, Mehlum A, Sandberg C, Bremnes B, Attramadal H. *J Biol Chem* 2000;275:17596. [PubMed: 10747877]
8. Innamorati G, Le Gouill C, Balamotis M, Birnbaumer MA. *J Biol Chem* 2001;276:13096. [PubMed: 11150299]
9. Zerial M, McBride H. *Nature Rev Mol Cell Biol* 2001;2:107. [PubMed: 11252952]
10. Pfeffer S. *Cell* 2003;112:507. [PubMed: 12600314]
11. Gorvel JP, Chavrier P, Zerial M, Greunberg J. *Cell* 1991;64:915. [PubMed: 1900457]
12. Gruenberg J, Maxfield FR. *Curr Opin Cell Biol* 1995;7:552. [PubMed: 7495576]
13. Gruenberg J. *Nat Rev Mol Cell Biol* 2001;2:721. [PubMed: 11584299]
14. Kornfeld S, Mellman I. *Annu Rev Cell Biol* 1989;5:483. [PubMed: 2557062]
15. Maxfield FR, McGraw TE. *Nature Rev Mol Cell Biol* 2004;5:121. [PubMed: 15040445]
16. Moore RH, Millman EE, Alpizar-Foster E, Dai W, Knoll BJ. *J Cell Sci* 2004;117:3107. [PubMed: 15190120]

17. Marchese A, Paing MM, Temple BR, Trejo J. *Annu Rev Pharmacol Toxicol* 2008;48:601. [PubMed: 17995450]
18. Fain JN, Buehrer B, Bahouth SW, Tichansky DS, Madan AK. *Metabolism* 2008;57:1005. [PubMed: 18555844]
19. Snyder EM, Philpot BD, Huber KM, Dong X, Fallon JR, Bear MF. *Nat Neurosci* 2001;4:1079. [PubMed: 11687813]
20. Jordens I, Marsman M, Kunjil C, Neefjes J. *Traffic* 2005;6:1070. [PubMed: 16262719]
21. Sonnichsen B, DeRenzis S, Nielsen E, Rietdorf J, Zerial M. *J Cell Biol* 2000;149:901. [PubMed: 10811830]
22. Marsh M, McMahon HT. *Science* 1999;285:215. [PubMed: 10398591]
23. Zeng J, Ren M, Gravotta D, De Lemos-Chiarandini C, Lui M, Erdjument-Bromage H, Tempst P, Xu G, Shen TH, Morimoto T, Adesnik M, Sabatini DD. *Proc Natl Acad Sci USA* 1999;96:2840. [PubMed: 10077598]
24. Lapierre LA, Kumar R, Hales CM, Navarre J, Bhartur SG, Burnette JO, Provance DW Jr, Mercer JA, Bahler M, Goldenring JR. *Mol Biol Cell* 2001;12:1843. [PubMed: 11408590]
25. Mammoto A, Ohtsuka T, Hotta I, Sasaki T, Takai Y. *J Biol Chem* 1999;274:25517. [PubMed: 10464283]
26. Prekeris R, Davies JM, Scheller RH. *J Biol Chem* 2001;276:38966. [PubMed: 11481332]
27. Hales CM, Griner R, Hobdy-Henderson KC, Dorn MC, Hardy D, Kumar R, Navarre J, Chan EK, Lapierre LA, Goldenring JR. *J Biol Chem* 2001;276:39067. [PubMed: 11495908]
28. Lindsay AJ, Hendrick AG, Cantalupo G, Senic-Matuglia F, Goud B, Bucci C, McCaffrey MW. *J Biol Chem* 2002;277:12190. [PubMed: 11786538]
29. Wallace DM, Lindsay AJ, Hendrick AG, McCaffrey MW. *Biochem Biophys Res Commun* 2002;299:770. [PubMed: 12470645]
30. Shirane M, Nakayama KI. *Science* 2006;314:818. [PubMed: 17082457]
31. Volpicelli LA, Lah JJ, Fang G, Goldenring JR, Levey AI. *J Neurosci* 2002;22:9776. [PubMed: 12427833]
32. Ducharme NA, Jin M, Lapierre LA, Goldenring JR. *Methods Enzymol* 2005;403:706. [PubMed: 16473632]
33. Mammoto A, Sasaki T, Kim Y, Takai Y. *J Biol Chem* 2000;275:13167. [PubMed: 10747849]
34. Miserey-Lenkei S, Waharte F, Boulet A, Cuif MH, Tenza D, El Marjou A, Raposo G, Salamero J, Héliot L, Goud B, Monier S. *Traffic* 2007;8:1385. [PubMed: 17725553]
35. Prekeris R, Klumperman J, Scheller RH. *Mol Cell* 2000;6:1437. [PubMed: 11163216]
36. Tarbutton E, Peden AA, Junutula JR, Prekeris R. *Methods Enzymol* 2005;403:512. [PubMed: 16473616]
37. Hickson GRZ, Matheson J, Riggs B, Maier VH, Fielding AB, Prekeris R, Sullivan W, Barr FA, Gould GW. *Mol Biol Cell* 2003;14:2908. [PubMed: 12857874]
38. Horgan CP, Oleksy A, Zhdanov AV, Lall PY, White IJ, Khan AR, Futter CE, McCaffrey JG, McCaffrey MW. *Traffic* 2007;8:414. [PubMed: 17394487]
39. Wells AL, Lin AW, Chen LQ, Safer D, Cain SM, Hasson T, Carragher BO, Milligan RA, Sweeney HL. *Nature* 1999;401:505. [PubMed: 10519557]
40. Biemesderfer D, Mentone SA, Mooseker M, Hasson T. *Am J Physiol Renal Physiol* 2002;282:F785. [PubMed: 11934687]
41. Buss F, Arden SD, Lindsay M, Luzio JP, Kendrick-Jones J. *EMBO J* 2001;20:3676. [PubMed: 11447109]
42. Katsura T, Gustafson CE, Ausiello DA, Brown D. *Am J Physiol* 1997;272:F817. [PubMed: 9227644]
43. Rink J, Ghigo E, Kalaidzikis Y, Zerial M. *Cell* 2005;122:735. [PubMed: 16143105]
44. von Zastrow M, Kobilka BK. *J Biol Chem* 1992;267:3530. [PubMed: 1371121]
45. Moore RH, Tuffaha A, Millman EE, Dai W, Hall HS, Dickey BF, Knoll BJ. *J Cell Sci* 1999;112:329. [PubMed: 9885286]
46. Ehlers MD. *Neuron* 2000;28:511. [PubMed: 11144360]

47. Lombardi D, Soldati T, Riederer MA, Goda Y, Zerial M, Pfeffer SR. *EMBO J* 1993;12:677. [PubMed: 8440258]
48. Innamorati G, Sadeghi H, Tran NT, Birnbaumer MA. *Proc Natl Acad Sci USA* 1999;95:2222. [PubMed: 9482866]
49. Gentzsch M, Chang XB, Cui L, Wu Y, Ozols VV, Choudhury A, Pagano RE, Riordan JR. *Mol Biol Cell* 2004;15:2684. [PubMed: 15075371]
50. Fan GH, Lapierre LA, Goldenring JR, Richmond A. *Blood* 2003;101:2115. [PubMed: 12411301]
51. Nedvetsky PI, Stefan E, Frische S, Santamaria K, Wiesner B, Valenti G, Hammer JA, Nielsen S, Goldenring JR, Rosenthal W, Klussmann E. *Traffic* 2007;2:110. [PubMed: 17156409]
52. Swiatecka-Urban A, Talebian L, Kanno E, Moreau-Marquis S, Coutermarsh B, Hansen K, Barnaby R, Karlson KH, Barnaby R, Cheney RE, Langford GM, Fukuda M, Stanton BA. *J Biol Chem* 2007;282:23725. [PubMed: 17462998]
53. Lise MF, Wong TP, Trinh A, Liu L, Kang R, Hines DJ, Lu J, Goldenring JR, Wang YT, El-Husseini A. *J Biol Chem* 2006;281:3669. [PubMed: 16338934]
54. Wang Z, Edwards JG, Riley N, Provance DW, Karcher R, Li X, Davidson IG, Ikebe M, Mercer JA, Kauer JA, Ehlers MD. *Cell* 2008;284:535. [PubMed: 18984164]
55. Chu BB, Ge L, Xie C, Zhao Y, Miao HH, Wang J, Li BL, Song BBL. *J Biol Chem* 2009;284:22481. [PubMed: 19542231]
56. Wu H, Nash JE, Zamorano P, Garner CC. *J Biol Chem* 2002;277:30928. [PubMed: 12050163]
57. Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J. *Science* 2010;327:1653. [PubMed: 20185685]

Figure 1. Internalized $\beta 1$ -adrenergic receptors localize to early endosomes

A, C. Time course for the association of internalized $\beta 1$ -AR with rab5a (A), or EEA1 (C). HEK-293 cells stably expressing equivalent amounts of WT $\beta 1$ -AR or S312A $\beta 1$ -AR were exposed to isoprenaline for 5 min, then to alprenolol. One culture dish was exposed to buffer for 5 min and then fixed (Δ). At the indicated time periods each slide was fixed, permeabilized and stained simultaneously with mouse monoclonal anti-Flag M2 IgG or rabbit polyclonal antibodies to either rab5a or EEA1. The primary antibodies were washed and the cells were incubated with Cy3-conjugated to goat anti-mouse IgG (red dot) or FITC-conjugated goat anti-rabbit IgG (green dot). Scale bar = 10 μ m. **B, D.** The percent of internal $\beta 1$ -AR structures labeling for each marker was quantified at the indicated time points by confocal microscopy following the protocol described in the “Materials and Methods” section. Data represent mean $\pm$ SEM from 4–6 cells and each experiment was replicated $n = 3$ times. P values <0.05 were denoted with one asterisk (*), while $p < 0.01$ or $p < 0.001$ were denoted with a double (**) or triple asterisk (***).

Figure 2. β 1-adrenergic receptors internalized in response to isoprenaline transiently colocalize with rab4a and the transferrin receptor

A, C. Time course for the association of internalized β 1-AR with rab4a (A), or Tfr (C). HEK-293 cells stably expressing equivalent amounts of WT β 1-AR or S312A β 1-AR were exposed to isoprenaline for 5 min, then to alprenolol. **A,** At the indicated time periods each slide was fixed, permeabilized and stained simultaneously with mouse monoclonal anti-Flag M2 IgG or rabbit polyclonal antibody to rab 4A. The primary antibodies were washed and the cells were incubated with FITC-labeled goat anti-mouse IgG (green dot) or Texas red-labeled goat anti-rabbit IgG (red dot). **C,** the slides were stained simultaneously with FITC-conjugated monoclonal anti-human Tfr IgG (green dot) and Cy3-conjugated monoclonal anti-Flag M2 IgG (red dot). **B, D,** The percent of internal β 1-AR structures labeling for each marker was quantified at the indicated time points by confocal microscopy following the protocol described in the “Materials and Methods” section. Data represent mean $\pm$ SEM from 4–6 cells and each experiment was replicated $n = 3$ times.

Figure 3. S312A β 1-adrenergic receptors internalized in response to isoprenaline colocalize with late endosomes/lysosomes

HEK-293 cells stably expressing equivalent amounts of WT β 1-AR or S312A β 1-AR were exposed to isoprenaline for 5 min, then to alprenolol. **A, C.** At the indicated time periods each slide was fixed, permeabilized and stained simultaneously with mouse monoclonal anti-Flag M2 IgG or rabbit polyclonal antibody to rab 9 or lamp1. The primary antibodies were washed and the cells in panel A, were incubated with FITC-conjugated goat anti-mouse IgG (green dot) or Texas red-conjugated goat anti-rabbit IgG (red dot), while those in panel C were stained simultaneously with FITC-conjugated monoclonal anti-human Tfr IgG (green dot) and Cy3-conjugated monoclonal anti-Flag M2 IgG (red dot). **B, D.** The time course for association of internalized S312A β 1-AR with rab9-(A), and Lamp1-(B) and percent colocalization between each β 1-AR and rab9-(C) or Lamp1-(D) were conducted as described in the legend of Fig. 1 and 2.

Figure 4. Isoprenaline accelerates the rate of degradation of surface biotinylated S312A $\beta 1$ -AR
A, HEK-293 cells stably expressing equivalent densities of WT or S312A $\beta 1$ -AR were surface biotinylated and then exposed to isoprenaline for the indicated times in the abscissa. At each time point, equal amounts of total protein cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-FLAG M2 IgG. **B**, The (density in each isoprenaline-treated sample)/[density in untreated samples) were calculated to determine the percentile loss of each $\beta 1$ -AR over time.

Figure 5. WT β_1 -adrenergic receptor internalized in response to isoprenaline colocalize with the recycling endosomal marker rab11

The time course for association of internalized WT β_1 -AR and S312A β_1 -AR with rab11, and percent colocalization between each β_1 -AR and rab11 were conducted as described in the legends of Fig. 1 and 2.

Figure 6. Overexpression of rab11S25N or knockdown off rab11a inhibits the recycling of agonist-internalized WT β 1-AR

A, HEK-293 cells co-expressing the WT β 1-AR with empty pcDNA vector [a-f], WT rab11a (g-l), dominant negative (S25N) rab11a (m-r), or constitutively active (Q70L) rab11a (s-x) were used to conduct the confocal recycling assay described in “Materials and Methods”. **B**, quantification of β 1-AR recycling kinetics. Pixels inside a 300-nm boundary in isoprenaline/acid-washed cells were set arbitrarily to 100% to indicate 100% internalization and the ratios in alprenolol treated cells were calculated and expressed as % for each time period. The $t_{0.5}$ for recycling was calculated as described in the “Material and Methods” section. The p values represent the difference in the distribution of the WT β 1-AR in control cells *versus* its distribution in mutant rab11-expressing cells. **C**, HEK-293 cells were transfected with at a 1:4 ratio of FLAG-tagged WT β 1-AR with scrambled shRNA in mir pcDNA 6.2 vector (a'–c'), shRNA to rab11a (d'–f') and shRNA to rab11b (g'–i'). These cells were used after 2 days to determine the effect of knockdown of rab11a or rab11b on recycling of the WT β 1-AR. **D**, HEK-293 cells were transfected with 4 μ g of scrambled shRNA, rab11a shRNA or rab11b

shRNA for 2d. Cell lysates from untreated, scr. shRNA or rab11a/b shRNA were probed by western blotting for rab11a/b expression. In these experiments 10 or 30 μ g of protein was probed with rab11a or rab11b, respectively. Knock down levels of rab11a/b were estimated by scanning densitometry. Each blot was stripped and probed with an antibody to β -actin to correct for minor differences in protein input levels.

Figure 7. Effect of knockdown of rab11-interacting proteins on recycling of the agonist-internalized WT- β 1-adrenergic receptors

HEK-293 cells were transfected with at a 1:4 ratio of FLAG-tagged WT β 1-AR and the indicated shRNA in mir pcDNA 6.2 vector. After 2 days all cells were labeled with FITC anti-FLAG M2 and then those cells in (a, d, g, j, and m) were fixed. The remaining plates were treated with isoprenaline for 30 min, followed by acid wash. Culture dishes representing images [b, e, h, k, n] were fixed. The other culture dishes were placed in media containing alprenolol for 60 min, fixed and imaged by confocal microscopy (images c, f, i, l, o). Data are representative from 6–10 cells and each experiment was replicated 3 times.

Figure 8. Effect of knockdown of the rab11-family of interacting proteins (FIPs) on recycling of the agonist-internalized WT- β 1-adrenergic receptors

A, HEK-293 cells were transfected with FLAG-tagged WT β 1-AR the indicated FIP shRNA in mir pcDNA 6.2 vector (Invitrogen) at a 1:4 ratio. After 2 days all cells were labeled with FITC anti-FLAG M2 antibody and then fixed (No treatment), or exposed to isoprenaline followed by acid stripping and fixation (ISO 30' acid wash). Cells exposed to isoprenaline followed by acid-stripping were placed in media containing alprenolol for 60 min and then fixed and imaged by confocal microscopy (Alprenolol 60 min). The data are representative from 6–10 cell and each experiment was replicated 3 times. Scale = 10 μ m. B, Cells that were transfected with WT β 1-AR and myosin VI shRNA were either fixed (*p*) or exposed to isoprenaline for 30 min (*q*) followed by acid stripping and fixation (*r*).