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Inhibitors of phosphoinositide 3-kinase cause defects in the postendocytic sorting of β_2 -adrenergic receptors

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Abstract

Phosphatidylinositol 3-kinase inhibitors have been shown to affect the endocytosis or subsequent intracellular sorting in various receptor systems. Agonist-activated β_2 -adrenergic receptors undergo desensitization by mechanisms that include the phosphorylation, endocytosis and degradation of receptors. Following endocytosis, most internalized receptors are sorted to the cell surface, but some proportion is sorted to lysosomes for degradation. It is not known what governs the ratio of receptors that recycle versus receptors that undergo degradation. To determine if phosphatidylinositol 3-kinases regulate β_2 -adrenergic receptor trafficking, HEK293 cells stably expressing these receptors were treated with the phosphatidylinositol 3-kinase inhibitors LY294002 or wortmannin. We then studied agonist-induced receptor endocytosis and postendocytic sorting, including recycling and degradation of the internalized receptors. Both inhibitors amplified the internalization of receptors after exposure to the β -agonist isoproterenol, which was attributable to the sorting of a significant fraction of receptors to an intracellular compartment from which receptor recycling did not occur. The initial rate of β_2 -adrenergic receptor endocytosis and the default rate of receptor recycling were not significantly altered. During prolonged exposure to agonist, LY294002 slowed the degradation rate of β_2 -adrenergic receptors and caused the accumulation of receptors within rab7-positive vesicles. These results suggest that phosphatidylinositol 3-kinase inhibitors (1) cause a misrouting of β_2 -adrenergic receptors into vesicles that are neither able to efficiently recycle to the surface nor sort to lysosomes, and (2) delays the movement of receptors from late endosomes to lysosomes.

Keywords

PI 3-kinase; β_2 -adrenergic receptors; rab7; lysosomes; endosomes

Introduction

Signaling by β_2 -adrenergic receptors (β_2 ARs) upon treatment with β_2 -agonists is attenuated by several distinct mechanisms. Phosphorylation of receptors by G-protein-coupled receptor (GPCR) kinases and protein kinases A and C [1–3] causes partial uncoupling from G-proteins.

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Similar to other GPCRs, phosphorylated β_2 ARs then bind with high affinity to β -arrestin, which fully uncouples receptors from G proteins [4] and also interacts with clathrin and adaptor proteins, thereby facilitating rapid receptor endocytosis [5,6]. Internalized receptors traffic through early endosomes, localizing with transferrin receptors [7] and rab5a [8] before returning to the cell surface (recycling). During prolonged exposure to agonists, β_2 ARs may also sort from early endosomes to lysosomes via late endosomes for degradation [9,10]. Receptor endocytosis and subsequent intracellular sorting events are thus critical regulators of surface β_2 AR number, thereby influencing the intensity of tissue responses to β -agonists used as therapeutic agents.

Given the importance of proper postendocytic sorting to maintain adequate surface receptor numbers, much effort has been made to discover intracellular factors that regulate the choice between receptor degradation and receptor recycling. An important advance in the study of protein sorting was the discovery that phosphatidylinositol 3-kinase (PI 3-kinase), which phosphorylates phosphatidylinositol to phosphatidylinositol 3-phosphate (PtdIns3P), is required for the transport of membrane proteins to the yeast vacuole [11]. Subsequent work with intact mammalian cells showed that pharmacologic inhibition of PI 3-kinase by the drugs LY294002 or wortmannin inhibits the intracellular trafficking of receptors, including the recycling of transferrin receptors [12–15] and the degradation of PDGF receptors [16,17]. A previous study also showed that wortmannin treatment reduces the recycling kinetics of the G protein-coupled angiotensin-1 receptor [18], indicating that postendocytic trafficking of GPCRs might be regulated by phosphoinositides. However, there is no information regarding the dependence of β_2 AR degradation or recycling on PtdIns3P production.

Since PI 3-kinase inhibitors affect the postendocytic sorting of other receptors, we sought to determine whether such compounds would affect the degradation or recycling of β_2 ARs. We thus treated HEK293 cells stably expressing β_2 ARs with PI 3-kinase inhibitors and then examined agonist-induced receptor endocytosis and postendocytic sorting, including the recycling and degradation of internalized receptors.

Materials and methods

Cells and reagents

12 β 6 cells, an HEK293 line expressing ~1 pmol/mg of hemagglutinin (HA)-tagged β_2 ARs, were obtained from B. Kobilka. A rabbit polyclonal antibody to the human β_2 AR C-terminus (H-20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody to the human Na⁺/K⁺-ATPase was obtained from Upstate Cell Signaling Solutions (Charlottesville, VA), and a monoclonal antibody mHA.11 against the HA tag from Covance (Berkeley, CA). Fluorescent secondary antibodies (goat anti-rabbit IgG-Texas Red and goat anti-mouse IgG-Alexa 488), and Alexa 594 holotransferrin, were purchased from Invitrogen-Molecular Probes (Eugene, OR). [³H]CGP12177 (33 Ci/mmol) was purchased from NEN Life Sciences (Boston, MA), (-) isoproterenol (ISO) bitartrate from ICN (Irvine, CA), and LY294002 from Biomol (Plymouth Meeting, PA). A construct expressing EGFP-rab7 was constructed by PCR amplification of a human rab7 cDNA and was subcloned into pEGFP-C1. FuGene 6 transfection reagent was obtained from Roche Applied Science. (Indianapolis, IN). All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Measurement of β_2 AR endocytosis

12 β 6 cells growing in 24-well clusters were equilibrated in medium containing LY294002 or vehicle (0.075% ethanol) for 30 min, then with ISO (10 μ M) in triplicate wells for varying times up to 20 min at 37°C. LY294002 or vehicle was present throughout the agonist incubation period. The clusters were rapidly chilled and the wells washed four times with ice-cold DMEM-

H (DMEM with 20 mM HEPES, pH 7.4). The cells were then incubated at 4°C for 90 minutes with 6 nM [³H] CGP-12177, a hydrophilic radioligand that selectively binds surface β_2 ARs. The cells were washed twice with cold DMEM-H, and lysed with 0.1% SDS, 0.1% NP-40 for counting by scintillation spectroscopy. In dose-response experiments, cells were treated with varying concentrations of LY294002 or wortmannin, then with 10 μ M ISO for 20 min prior to measuring surface receptors. In time-course experiments, LY294002 was used at 50 μ M and incubations with ISO varied up to 20 min. Nonspecific binding was assessed by including 1 μ M propranolol in parallel binding incubations, and was always <3% of the total binding.

Measurement of β_2 AR recycling

The recycling assay measured the rate at which internal receptors returned to the cell surface after removal of agonist. Cells growing in 24-well clusters were first treated with vehicle or with LY294002 for 30 min, then with 10 μ M ISO in triplicate wells for 20 min to obtain a steady-state level of internalized receptors. LY294002 (50 μ M) or vehicle was present throughout the agonist incubation period. The cells were washed rapidly three times with warm medium, and then incubated at 37°C in medium with vehicle or LY294002 for varying times to allow recycling. The clusters were chilled on ice, and surface receptors were measured by [³H]CGP-12177 binding as described above. The first-order recycling rate constant (k_r) was determined using nonlinear regression to calculate the rate at which internalized receptors recycle to the maximal extent, as described by us previously [19]. The data were also plotted without modeling to show a time course of internalization and return of receptors to the cell surface.

Colocalization of β_2 ARs and EGFP-rab7

12 β 6 cells were grown on glass coverslips in 6-well clusters to 50–60% confluence, then were transiently transfected for 24 h with EGFP-rab7 DNA using FuGene 6 transfection reagent. For colocalization, the cells were equilibrated for 30 min in fresh media containing either vehicle or 50 μ M LY294002, then were treated with 0.1 mM ascorbate, 1 mM thiourea (AT: used to prevent ligand oxidation during prolonged incubations) or AT + 10 μ M ISO for 1, 2, or 4 h. The cells were washed with ice-cold 1X PBSS (PBS with 1.2% sucrose), fixed with 4% paraformaldehyde at 4°C, then washed at room temperature with 1X PBSS (PBS with 0.1% sucrose). Next, the cells were incubated with 0.34% L-lysine/0.05% Na-periodate, permeabilized with 0.2% Triton X-100, then blocked with 10% normal goat serum. The cells were incubated in primary polyclonal rabbit antibody (4 μ g/ml) that recognizes the carboxyl terminus of the β_2 AR (anti- β_2 AR-CT) for 1 h, then washed with 1X PBSS and incubated for 30 min with goat anti-rabbit secondary antibodies (1:2000 dilution) conjugated to Alexa 594. After additional washes, the cover slips were mounted on slides with Mowiol containing 2.5% 1,4-diazabicyclo-[2,2,2]octane. Images were captured using an inverted Olympus IX70 microscope fitted with a 100X lens (NA = 1.4) and a Quantix EEV57 back-thinned CCD camera.

Quantification of imaging analysis

Image stacks of thirty-two 0.2 μ m optical sections were collected and deconvolved using software from ISee Imaging (Raleigh, NC), which uses a constrained iterative procedure and a predetermined point spread function [20]. Colocalization of EGFP-rab7 with Alexa594-labeled β_2 ARs was quantified using Metamorph (Universal Imaging Corp., Downingtown, PA). For each cell, three images from the top, middle and bottom of the cell (namely stacks 8, 16 and 24 from the 32 images in the stack) were used to quantify colocalization. The colocalization was measured by the overlap of the green and red fluorophores with each other, which was calculated by Metamorph® as % integrated for each image stack. Both red overlap with green and green overlap with red gave very similar patterns. For each time point of agonist

exposure, 6–9 cells from two separate experiments were chosen for analysis on the basis of their displaying a vesicular EFGP-rab7 morphology and minimal EGFP-rab7 expression levels, to minimize any effects of rab7 overexpression.

Transferrin recycling

12β6 cells were incubated in DMEM + 0.1% BSA for 60 min, with LY294002 (50 μM) or vehicle present during the last 30 min at 37°C. Alexa594 holotransferrin (50 μg/ml) and ISO (10 μM) were then added for 30 min. The cells were quickly washed twice with 37°C PBS, then incubation was continued at 37°C with DMEM + 0.1% BSA, 1 μM propranolol, 50 μg/ml holotransferrin and either LY294002 or vehicle for another 40 min. The cells were then fixed and labeled with anti-β₂AR-CT primary and goat anti-rabbit Alexa 488 secondary antibodies, as described above.

Measurement of β₂AR degradation using surface biotinylation

12β6 cells were grown in 6-well plates, then were surface biotinylated using impermeant non-cleavable EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL) at 1 mg/ml for 30 min at room temperature. Biotinylation was followed by four washes with warm PBS to remove excess reagent. The cells were then incubated in medium for varying times up to 8 h with AT only or AT plus 10 μM ISO. The cells were collected by centrifugation and then lysed in 250 μl DDM lysis buffer (20 mM HEPES, 300 mM NaCl, 0.8% n-dodecyl-β-D-maltoside [DDM], 5 mM EDTA) on ice for 15 min. The samples were centrifuged at 13,000 Xg for 15 min 4°C and the supernatants saved. Streptavidin-agarose beads (50 μl) were added to the supernatant and the mixture was rotated for 1 h at 4°C. The volume of beads used was predetermined to be in excess of that needed to recover >90 % of the intact receptors remaining in the cell lysate. The beads were recovered by centrifugation and then washed with 500 μl of cold DDM lysis buffer. The biotinylated proteins were eluted from the beads in 50 μl of SDS sample buffer. The samples were then heated at 60°C for 15 min, centrifuged to remove the resin and the supernatant, then treated with peptide:N-glycosidase F (New England Biolabs, Beverly, MA) for 1–2 h at 37°C to deglycosylate receptors. Equivalent protein amounts (based on the streptavidin bead supernatants) were electrophoresed on 10% Tris-glycine gels and electroblotted. The membranes were probed with rabbit anti-β₂AR-CT antibodies (1:1000), then washed and incubated with goat anti-rabbit IgG secondary antibodies (1:80,000). The bound antibodies were detected by chemiluminescence and then visualized and quantified with a FluorChem 8800 CCD imaging system equipped with automatic bloom detection (Alpha Innotech Corporation, San Leandro, CA). Receptors were quantified in the linear range of detection, as determined by immunoblotting of serial dilutions of 12β6 cell extracts. The receptor band intensity was normalized to the band intensity of cell surface Na⁺/K⁺-ATPase used as a loading control. At each time point, the normalized levels of receptors remaining after incubation with AT + ISO were divided by the normalized levels remaining after incubation with AT only.

Results

Treatment with PI 3-kinase inhibitors augments agonist-induced β₂AR internalization

To determine the effect of PI 3-kinase inhibitors on the cellular trafficking of agonist-activated β₂ARs, various studies were performed on HEK293 cells stably expressing β₂ARs (12β6 cells). Initially, we determined the concentration and effect of the PI3-kinase inhibitor, LY 294002 on receptor internalization. Cells were treated with varying concentrations of LY294002, followed by a saturating concentration of a full agonist, isoproterenol (ISO) for 20 min as detailed in the methods. Radioligand binding studies showed that treatment with LY294002 augmented agonist-induced receptor internalization in a dose-dependent manner, as indicated by lower levels of surface receptors after agonist treatment (Fig. 1A). These results were further

confirmed in an independent line of HEK293 cells expressing β_2 ARs. We also found that these results are not specific to LY294002 since similar results were obtained with the structurally unrelated PI 3-kinase inhibitor wortmannin (data not shown). The decrease in surface receptors required the presence of agonist (Fig. 1A, inset, and Fig. 1 legend). A 50 μ M concentration of LY294002 was used in subsequent studies since it is known to be stable during prolonged incubations [21]. We also verified that LY294002 is stable during prolonged incubation, as medium from cells treated for 24 h with the LY294002 augmented agonist-induced β_2 AR internalization when applied to fresh 12 β 6 monolayers (data not shown).

Further analysis of β_2 ARs by immunofluorescence microscopy of LY294002-treated cells fixed after 20 min of ISO exposure showed that internalized β_2 ARs localized to relatively enlarged endocytic vesicles compared to vehicle-treated cells (Fig. 1B), and similar results were obtained with wortmannin-treated cells (not shown).

The apparent increase in receptor internalization could be due to an increased rate of endocytosis or a decreased rate of β_2 AR recycling [19]. To determine the effect of PI 3-kinase inhibitors on the rate of receptor endocytosis, a time-course of receptor internalization following agonist addition was performed. In the presence of 50 μ M LY294002, surface receptor levels reached a lower steady-state level compared with vehicle-treated cells (Fig. 2A), consistent with the dose-response data (Fig. 1A), and reflecting augmented receptor internalization. The initial endocytic rate constant was estimated by performing a semi logarithmic transformation of the data describing surface receptor loss over the first three min of endocytosis, before recycling or other sorting events are likely to have occurred to any significant extent. The slopes of the lines produced in these plots are equivalent to the first-order endocytic rate constants [19]. The initial rate of agonist-induced β_2 AR endocytosis is the same in the presence of vehicle or LY294002 (Fig. 2B, legend).

The extent but not the rate of β_2 AR recycling is decreased by LY294002 treatment

Since our results show that LY294002 does not affect the initial rate of β_2 AR endocytosis, we next measured the rate and extent of receptor recycling. 12 β 6 cells were treated with agonist for 20 min to achieve a steady-state fraction of internal receptors in the presence of vehicle or LY294002. The cells were then washed extensively and incubated at 37°C for varying times in the presence of vehicle or LY294002, prior to measuring surface receptor levels using [3 H] CGP12177. The recycling data in Fig. 3A were transformed so that the curves describe the rate of loss of internal receptors via their recycling to the plasma membrane. The most striking difference between the recycling curves (Fig. 3A) is that the number of nonrecyclable receptors is increased in LY294002-treated cells. The plateau values of the recycling curves were determined by nonlinear regression, and were significantly different ($40.0 \pm 2.5\%$ for LY294002-treated cells and $23.8 \pm 2.7\%$ for vehicle-treated control cells; $P = 0.047$). In contrast, the first-order recycling rate constants, as determined by nonlinear regression, did not differ significantly (Fig. 3, legend). When plotted as time course to show the return of receptors to the surface after the removal of agonist, the recycling curves are very similar, and differ mainly due to the differing extents of receptors internalization (Fig. 3B).

The internal receptors that remained after 40 min of recycling were readily visualized by immunofluorescence microscopy in cells treated with LY294002 compared with vehicle-treated cells (Fig. 3C). Approximately 90% of the cells showed such intracellular receptors, and similar results were obtained using wortmannin (data not shown).

The effect of LY294002 on transferrin recycling

Inhibition of PI 3-kinase reduces the rate of transferrin recycling in some studies [22], and there is overlap in the endosome compartment between internalized β_2 ARs and transferrin [7]. We

thus determined whether the extent of transferrin and β_2 AR recycling were similarly inhibited by LY294002 in 12 β 6 cells under our experimental conditions. A pulse-chase procedure was performed, where we fed cells with fluorescent transferrin during a 30 min exposure to ISO, then washed and incubated for 40 min in the presence of 1 μ M propranolol and excess unlabeled holotransferrin to permit the recycling of both β_2 ARs and transferrin receptors. Although β_2 ARs were still trapped inside cells after 40 min of recycling in the presence of LY294002, transferrin recycling under these experimental conditions was relatively unaffected (Fig. 4).

β_2 AR degradation is slowed by LY294002

PI 3-kinase inhibitors reduce the rate of degradation of some internalized growth factor receptors [16,17]. During prolonged exposure to agonist, β_2 ARs undergo downregulation, a loss of receptors that correlates with their movement through late endosomes and into lysosomes [9,10]. Given that our results above show that PI 3-kinase inhibitors cause the intracellular retention of β_2 ARs, we expected that there might be changes in the rate of β_2 AR degradation or a change in the final extent of degradation. We treated 12 β 6 cells for up to 8 h with vehicle or LY294002 in the presence or absence of agonist. There was a significant reduction in the initial rate of β_2 AR degradation, though the final extent of β_2 ARs degraded after 8 h was similar in both LY294002 and vehicle-treated cells (Fig. 5).

Enhanced colocalization of β_2 ARs and EGFP-rab7 after treatment with LY294002

Our β_2 AR degradation studies showed that the degradation of β_2 ARs was delayed in the presence of LY294002, which implies that receptors were eventually sorted to lysosomes. We next sought to determine whether the reduction in the initial β_2 AR degradation rate caused by LY294002 was due to a delay in the sorting of receptors to late endosomes, an intermediate compartment between early endosomes and lysosomes. We transfected 12 β 6 cells with EGFP-rab7 to mark the late endosomal compartment, then treated the cells with LY294002 or vehicle for 30 min prior to the addition of ISO for 0–4 h. The cells were fixed and labeled with anti- β_2 AR antibodies, then imaged by deconvolution fluorescence microscopy. As shown in Fig. 6A, β_2 ARs rapidly accumulated within EGFP-rab7-containing vesicles in LY294002-treated cells compared with vehicle-treated cells. The imaging data are quantified in Fig. 6B, as a percentage integrated of overlap between the two fluorophores (red over green). The results are similar whether red over green or green over red are measured (Table 1).

To provide evidence against nonspecific colocalization of intracellular markers caused by LY294002, we transfected cells with EGFP-rab5a and treated with agonist for 2 h. Inhibition of PI 3-kinase did not cause significant colocalization of rab5a and β_2 ARs (Fig. 6C).

Discussion

Our major finding is that inhibitors of PI 3-kinase disrupt the postendocytic sorting of β_2 ARs. This is manifested in three ways: (1) internalized β_2 ARs are prevented from recycling normally to the cell surface (Fig. 3C); (2) the rate of agonist-induced receptor degradation is slowed (Fig. 5); and (3) β_2 ARs accumulate in EGFP-rab7-positive vesicles (Fig. 6). The effect on recycling is different for β_2 ARs compared with transferrin receptors (Fig. 4), as the latter appear to enter a recycling pathway that is insensitive to the inhibitor during a simultaneous 30 min period of internalization for both receptors. All of these effects are caused by a concentration of PI 3-kinase inhibitor (LY294002) that has no detectable effect on the initial rate of agonist-induced β_2 AR endocytosis (Fig. 2).

Although PI 3-kinase inhibitors reduce the number of recyclable β_2 ARs, the intrinsic or default rate of receptor recycling is not detectably altered (Fig. 3A and B). This suggests that while PI 3-kinase inhibitors inhibit receptor sorting into the recycling pathway, these drugs do not appear

to significantly affect the machinery of the β_2 AR recycling pathway itself. Also, after recycling for 40 min in the absence of agonist and in the presence of LY294002 or wortmannin, a significant number of β_2 ARs become trapped within an intracellular compartment (Fig. 3C). This compartment seems to consist of aberrant endosomes, because these internal receptors did not localize with several markers of intracellular compartments, namely EGFP-galactosyltransferase (trans-Golgi network), EGFP-rab5 (early endosome), EGFP-rab11 (perinuclear recycling endosome), EGFP-rab7 (late endosome) or LAMP-1 (lysosome) (data not shown). Only after prolonged and continuous agonist exposure driving receptor endocytosis in the presence of LY294002 was β_2 AR colocalization with EGFP-rab7 significant (Fig. 6).

In previously published studies, treatment of cells with wortmannin or LY294002 reduced transferrin recycling rates [14,15,22], or had minimal effects [13,23], while the recycling kinetics of AT1 angiotensin receptors were reduced [18]. In contrast, LY294002 had no measurable effect on the recycling kinetics (k_r) of β_2 ARs (Fig. 3A, B), although the extent of β_2 AR recycling was reduced due to the intracellular retention of receptors (Fig. 3C). In comparing the recycling of transferrin and β_2 AR, it should be noted that our transferrin labeling protocol was sufficiently prolonged such that most of the label had entered recycling endosomes, which are known to be refractory to the effects of PI 3-kinase inhibitors. However, our data do suggest that transferrin and β_2 ARs do not follow identical intracellular itineraries during recycling.

In terms of receptor trafficking, GPCRs are classified as class A, which rapidly and completely recycle, and class B, which do not [24]. A further distinction is that β -arrestin internalizes into punctate vesicles with class B receptors, but do not with class A. It is unlikely that PI 3-kinase inhibition causes the class A β_2 AR to behave like a class B receptor, because we do not observe punctate intracellular β -arrestin in the presence of LY294006 after treatment with the agonist, ISO (data not shown).

In addition to reducing the extent of β_2 AR recycling, LY294002 significantly reduces the rate of agonist-induced degradation as assessed by a cell-surface biotinylation assay (Fig. 5). This finding is consistent with previous studies on the effects of PI 3-kinase inhibitors on the degradation of other signaling receptors, such as the PDGF receptor [16] and the EGF receptor [25,26]. There are at least three PI 3-kinases in mammalian cells, and the most commonly used PI 3-kinase inhibitors act on all three [27]. Evidence from other studies implicates the type III PI 3-kinase hVps34 in controlling traffic to lysosomes [28,29]. Whether hVps34 is the PI 3-kinase responsible for controlling β_2 AR sorting to lysosomes will require additional studies.

PtdIns3P localizes to specific microdomains of endosomes [30,31], recruiting proteins that contain PtdIns3P-binding domains and control receptor sorting. The two most important PtdIns3P-binding domains are the FYVE (Fab1, YOTB, Vac1 and EEA1) zinc finger domain [32] and the PH (phox-homology) domain [33]. Proteins with these domains play a role in sorting various ubiquitinated receptors from late endosomes to lysosomes. Agonist induced β_2 AR degradation could occur by this mechanism, because these receptors are ubiquitinated after agonist activation, and removal of all potential sites of β_2 AR ubiquitination reduces receptor downregulation [34]. Our finding that LY294002 inhibits β_2 AR degradation supports this possibility. Further studies are needed to identify those PtdIns3P-binding proteins that are needed for the proper postendocytic sorting of β_2 ARs.

In the presence of PI 3-kinase inhibitors and prolonged agonist activation, the reduction in β_2 AR degradation was accompanied by an accumulation of receptors in EGFP-rab7-containing vesicles. Rab7 localizes to late endosomes [35] and controls the traffic of membrane proteins from the early endosome to lysosomes [36-41]. The enhanced colocalization of β_2 ARs and

EGFP-rab7 in late endosomes in the presence of LY294002 suggests that PI 3-kinase is required for the transport of membrane proteins from late endosomes to lysosomes. The delay we observe in agonist-induced β_2 AR degradation (Fig. 5) is consistent with this interpretation. The molecular mechanisms underlying rab7 action are not clear at present, however a recent study shows that rab7 binds to hVps34 (a PI 3kinase) and localizes it to late endosomes [29].

PI 3-kinase inhibitors did not affect the initial rate of β_2 AR endocytosis in our system (Fig. 2B). This is consistent with other studies where the endocytosis of transferrin receptors [14, 15], β_2 ARs [42] and AT1 angiotensin receptors [18] were measured in the presence of concentrations of wortmannin low enough to prevent nonspecific effects. Other studies have suggested that wortmannin inhibits agonist-induced β_2 AR endocytosis, and that agonist-dependent recruitment of a PI 3-kinase to the plasma membrane is important for this process [43]. The discrepancy is most likely due to the different β_2 AR expression methods used. Our study was performed in stably transfected HEK293 cells, which have a much higher initial rate of β_2 AR endocytosis and higher coupling efficiencies than the transiently transfected HEK293 cell systems utilized in the other studies [43]. Furthermore, we observed the same results on endocytosis using an independently derived stably transfected line of HEK293 cells (data not shown). Therefore, it does not appear that cell line variation explains the difference between our study and those previously published. An inhibitory effect of PI 3-kinase inhibition on β_2 AR endocytosis may be much more apparent when the endocytic rate constant is low to begin with, and when endocytic machinery is near saturation due to very high levels of receptor expression due to transient transfection [44]. We cannot rule out that PI 3-kinase inhibitors produce a slight reduction of endocytosis in our stably transfected cells that is not detectable by our methods. However, we can clearly conclude from our study that PI3-kinase inhibitors have a much more prominent effect on the postendocytic sorting of β_2 ARs than on β_2 AR endocytosis per se. Our data suggest an important role for PI 3-kinases in the sorting of β_2 ARs between the recycling and degradative pathways.

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Figure 1. LY294002 augments the decrease in the number of surface $\beta_2\text{AR}$ s caused by exposure to agonist

(A). 12 β 6 cells were treated with varying concentrations of LY294002 for 30 min, then with 10 μM ISO for 20 min followed by chilling to 4°C. Surface receptor levels were measured by binding to [^3H]CGP12177 as described in the Materials and Methods. (*) Significantly different from vehicle control ($P < 0.05$; $N=3$), one-way ANOVA, Dunnett's multiple comparisons post-test. A one-way ANOVA with a post-test for linear trends is also significant ($P < 0.0001$). **Inset.** 12 β 6 cells were treated for 30 min with LY294002 (LY- 50 μM), wortmannin (WT- 100nM), ethanol vehicle (EtOH – 0.075%), DMSO vehicle (DMSO – 0.1%), or not treated, then chilled and incubated on ice with [^3H]CGP 12177 as described in the Materials and Methods. Compared to the untreated control, the percentage of bound radioligand remaining on the surface was LY, 93.3 ± 3.0 ; WT, 86.12 ± 8.32 ; Eth, 91.7 ± 3.49 ; DMSO, 89.32 ± 5.61 . No pair of values was significantly different ($P > 0.05$, one-way ANOVA, Tukey's Multiple Comparison Test). (B). 12 β 6 cells were treated with 50 μM LY294002 or vehicle for 30 min, then with 10 μM ISO for 20 min followed by fixation with 4% paraformaldehyde and labeling with antibodies against the $\beta_2\text{AR}$ HA-tag. The cells were imaged by deconvolution immunofluorescence microscopy. Scalebar = 10 μm .

Figure 2. LY294002 does not affect the initial rate of β_2 AR endocytosis

12 β 6 cells were treated with 50 μ M LY294002 or vehicle for 30 min, then for varying times with 10 μ M ISO followed by chilling to 4°C. Surface receptor levels were measured by binding to [3 H]CGP12177 as described in the Materials and Methods. **(A)** Time-course of internalization, modeled according to Morrison et al [19]. The calculated plateau values, which represent the maximal fraction of receptors left on the cell surface, were 0.48 ± 0.01 for vehicle-treated control cells, and 0.32 ± 0.03 for LY294002-treated cells ($P = 0.0097$; $N=3$); **(B)** A plot of the natural logarithm of the surface receptor fraction remaining during the first three min after addition of agonist. (■) Vehicle; (□) LY294002. For vehicle treated cells, the slope of the line is -0.15 ± 0.02 , and for LY294002-treated cells, -0.15 ± 0.01 , representing first order rate constants of 0.15 min^{-1} for both vehicle and LY294002-treated cells.

Figure 3. LY294002 treatment inhibits the recycling of β_2 ARs

(A). 12 β 6 cells were treated with 50 μ M LY294002 or vehicle for 30 min, then with 10 μ M ISO for 20 min. The cells were washed extensively and returned to medium for varying times in the presence of 50 μ M LY294002 or vehicle. After chilling on ice, the cells were incubated with [3 H]CGP12177 to selectively label surface receptors. The fractional increases in surface receptors as a function of time after agonist removal were transformed to allow the plotting of the loss of internal receptors. The plots were then modeled using a first-order exponential function as described in Morrison et al [19] to obtain a plateau value for the number of receptors that fail to recycle, and the first order recycling rate constant. (■) Vehicle; (□) LY294002. The plateau values of the recycling curves were $40.0 \pm 2.5\%$ for LY294002-treated cells and 23.8

$\pm 2.7\%$ for vehicle-treated control cells ($P = 0.047$; $N=34$). The recycling rate constants were $0.107 \pm 0.014 \text{ min}^{-1}$ for vehicle-treated cells and $0.085 \pm 0.012 \text{ min}^{-1}$ for LY294002-treated cells (the rates are not significantly different). **(B)**. Recycling was plotted as a time course showing the return of receptors to the cell surface. **(C)**. Immunofluorescence imaging of recycled $\beta_2\text{ARs}$. *Veh*: 12 β 6 cells were treated with vehicle alone for 30 min, followed by 10 μM ISO for 20 min. The cells were then washed and incubated at 37°C in the presence of vehicle. Recycling was stopped after 40 min by fixation with 4% formaldehyde. *LY*: 12 β 6 cells were treated with 50 μM LY294002 for 30 min, followed by 10 μM ISO for 20 min. The cells were then washed and incubated at 37°C in the continuing presence of 50 μM LY294002. Recycling was stopped after 40 min by fixation with 4% formaldehyde. The fixed cells were labeled with antibodies against the $\beta_2\text{AR}$ C-terminus and imaged by conventional epifluorescence microscopy. Scalebar, 10 μm .

Figure 4. The effect of LY294002 treatment on the recycling of transferrin

12β6 cells were treated with 50 μM LY294002 or vehicle for 60 min in DMEM + 0.1% BSA at 37°C. ISO (10μM) and 50 μg/ml Alexa 594-holotransferrin were then added for 30 min at 37°C. The medium was removed and the cells were washed twice with PBS, and then were incubated for 40 min at 37°C with 1 μM propranolol and 50 μg/ml holotransferrin with either vehicle or 50 μM LY294002. The cells were fixed and then imaged using deconvolution fluorescence microscopy. The experiment was performed twice with similar results.

Figure 5. LY294002 delays agonist-induced degradation of β_2 ARs

12 β 6 cells were incubated for 30 min with 50 μ M LY294002 or vehicle, then surface biotinylated as described in Materials and Methods. The cells were incubated up to 8 h in medium with or without 10 μ M ISO and with 50 μ M LY294002 or vehicle. The cells were lysed and biotinylated proteins were recovered with streptavidin agarose beads. After elution from the beads, the proteins were deglycosylated and immunoblotted with antibodies to the β_2 AR C-terminus and to the cell-surface Na⁺,K⁺ATPase (ATPase). Blots were imaged and quantified using a CCD camera system as described in Materials and Methods. (A). Representative blots, showing deglycosylated β_2 ARs migrating at ~35 kDa, and Na⁺,K⁺

ATPase migrating at ~100 kDa. **(B).** Plot of the density of the receptor bands corrected for incubation after the same time period with no agonist present, and normalized to the Na⁺,K⁺ ATPase signal. The error bars show the range of two determinations.

Figure 6. LY294002 increases β_2 AR and EGFP-rab7 colocalization after prolonged agonist treatment

12 β 6 cells were transfected with a plasmid expressing EGFP-rab7 for 24 h, then treated with 50 μ M LY294002 or vehicle for 30 min prior to the addition of 10 μ M ISO + AT. (A) After 1–4 h of agonist exposure, the cells were fixed and then labeled with antibodies to the β_2 AR C-terminus (Alexa 594 secondary). Red, β_2 AR; Green, EGFP-rab7. The labeled cells were imaged by deconvolution microscopy as described in Materials and Methods. *Insets*: Two-fold magnifications of boxed areas in the panels. Scale bar = 10 μ m. (B) Quantification of β_2 AR and EGFP-rab7 overlap during prolonged agonist exposure. The overlap between β_2 AR and EGFP-rab7 (red over green) at various times following the addition of AT alone or AT + ISO was quantified using Metamorph as described in the Materials and Methods. The Y-axis is the percentage of β_2 AR labeling that overlaps with EGFP-rab7. (*) Significantly different from vehicle control: 1 h, $P < 0.0001$; 2 h, $P < 0.0007$; 4 h, $P < 0.005$. (C) The overlap between EGFP-rab5a and β_2 ARs after 2 h of agonist exposure. LY, LY294002; Veh, vehicle. 12 β 6 cells were transfected, treated and imaged as described above in (A), except that anti-HA antibody mHA. 11 was used to detect receptors.

Table 1Colocalization of EGFP-rab7 (green) and β_2 AR (red)

Red over green ISO, h	Vehicle, %	N	LY294002, %	N
0	2.11 $\pm$ 0.82	9	6.83 $\pm$ 1.38	6
1	10.59 $\pm$ 2.29	8	46.65 $\pm$ 3.83	8
2	17.60 $\pm$ 4.84	9	50.47 $\pm$ 6.11	9
4	18.05 $\pm$ 3.62	9	53.74 $\pm$ 7.34	9
Green over red ISO, h	Vehicle, %	N	LY294002, %	N
0	2.57 $\pm$ 0.78	9	9.21 $\pm$ 1.23	6
1	9.94 $\pm$ 2.64	8	43.09 $\pm$ 4.42	8
2	15.75 $\pm$ 2.35	9	43.99 $\pm$ 4.62	9
4	18.08 $\pm$ 3.67	9	49.30 $\pm$ 6.38	9