Agonist-induced sorting of human β2-adrenergic receptors to lysosomes during downregulation

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SUMMARY

During prolonged exposure to agonist, β2-adrenergic receptors undergo downregulation, defined by the loss of radioligand binding sites. To determine the cellular basis for β2-adrenergic receptor downregulation, we examined HEK293 cells stably expressing β2-adrenergic receptors with an N-terminal epitope tag. Downregulation was blocked by leupeptin, a cysteine protease inhibitor, but not by pepstatin, an inhibitor of aspartate proteases. Immunofluorescence microscopy of cells treated with agonist for 3-6 hours in the presence of leupeptin showed β2-adrenergic receptors, but not transferrin receptors, localizing with the lysosomal protease cathepsin D, and with lysosomes labeled by uptake of a fluorescent fluid-phase marker. No localization of β2-adrenergic receptors with lysosomal markers was observed in the absence of leupeptin, most likely due to proteolysis of the epitope. The proton pump inhibitor, bafilomycin A1, significantly inhibited this agonist-induced redistribution of β2-adrenergic receptors into lysosomes, causing receptors to accumulate in the rab11-positive perinuclear recycling compartment and slowing the rate of β2-adrenergic receptor recycling. Control experiments showed that leupeptin had no nonspecific effects on the cellular trafficking of either β2-adrenergic receptors or transferrin receptors. Although cAMP alone caused a small decline in receptor levels without redistributing β2-adrenergic receptors from the plasma membrane, this effect was additive to that seen with agonist alone, suggesting that agonist-induced β2-adrenergic receptor downregulation resulted largely from cAMP-independent mechanisms. These results indicate that during agonist-induced downregulation, a significant fraction of β2-adrenergic receptors are specifically sorted to lysosomes via the endosomal pathway, where receptor degradation by cysteine proteases occurs. These results provide a cellular explanation for the loss of radioligand binding sites that occurs during prolonged exposure to agonist.

Key words: β2-adrenergic receptor, Lysosome, Downregulation, Laser confocal microscopy, rab11, Bafilomycin A1

INTRODUCTION

G-protein coupled receptors (GPCRs) comprise an extended family of cell surface proteins that transduce signals from diverse kinds of extracellular stimuli to intracellular effector systems. A particularly well-studied GPCR is the β2-adrenergic receptor (β2AR), which is an important target for catecholamine hormones in physiologic actions such as the relaxation of vascular and airway smooth muscle. Agonist-bound β2ARs work principally by activating adenylyl cyclase through a coupled stimulatory G-protein (Gs), causing a rise in the intracellular levels of cAMP, a second messenger that eventually mediates most downstream physiological effects (Liggett, 1997).

The signaling capacity of β2ARs is attenuated by desensitization processes involving several distinct, but overlapping mechanisms. Within seconds of agonist binding, β2ARs are rapidly phosphorylated by G-protein coupled receptor kinases that recognize only agonist-bound receptors (Ferguson et al., 1997), and also by protein kinases A and C (Yuan et al., 1994), resulting in a functional uncoupling of receptors from Gs. A second rapidly occurring event, receptor endocytosis into endosomes, physically uncouples β2ARs from Gs (Ferguson et al., 1997; Morrison et al., 1996) and transports receptors to an early endosome compartment containing both transferrin receptors (von Zastrow and Kobilka, 1992) and the ras-related GTPase rab5 (Moore et al., 1995). β2ARs may be dephosphorylated at some point during their intracellular trafficking by a membrane-associated protein phosphatase 2A-like enzyme, following which receptors reappear on the cell surface in a fully sensitized state (Krueger et al., 1997; Yu et al., 1993). The kinetics of receptor internalization are relatively rapid (t1/2 < 3 minutes), as is recycling back to the cell surface (t1/2 < 8 minutes) (Morrison et al., 1996).

A third desensitization mechanism, downregulation, occurs during prolonged agonist treatment (at least several hours) and results in the loss of radioligand binding sites. In contrast to
rapid desensitization events (Ferguson et al., 1997), the mechanism of downregulation is poorly understood. At steady-state, the total number of β2ARs should be a function of the rates of receptor synthesis and loss of radioligand binding sites, either of which in principal could be influenced by agonist. Significant levels of receptor downregulation can be observed in cells treated with cell permeable analogs of cAMP or by activators of adenylyl cyclase, largely as a result of increased mRNA degradation (Bouvier et al., 1989). β2AR mRNA levels also are reduced following agonist treatment in some systems (Collins et al., 1992; Hadcock and Malbon, 1988), though not in others (Kelsen et al., 1997), suggesting that agonist may reduce the synthesis of β2ARs. However, this effect would be significant only if the half-lives of β2ARs themselves were relatively short, and available evidence indicates in fact that receptors are long-lived. Estimates of β2AR half-life, determined by measuring the rate of receptor restoration after blockade by an irreversible antagonist, suggests a basal half-life of up to 30 hours (Mahan and Insel, 1986), while the half time for downregulation is considerably shorter (3-10 hours) (Pittman et al., 1984). The implication is that while the rate of β2AR synthesis may be affected by agonist, there must also be significant receptor degradation.

For some signal-transducing receptors, the sequence of events during downregulation is understood in considerable detail. For example, ligand-bound epidermal growth factor receptors internalize into early endosomes before rapidly trafficking to lysosomes (within about 30 minutes), as assessed by detailed microscopic examination and subcellular fractionation (Chang et al., 1993). Also, the ligand-induced degradation of these receptors has been directly measured by biochemical procedures, and correlates temporally with lysosomal trafficking (Beguinot et al., 1984; Stoscheck and Carpenter, 1984). In contrast, there is little information about the intracellular fates of β2ARs during chronic agonist exposure. In recent reports (Gagnon et al., 1998; Kallal et al., 1998), during agonist-induced downregulation, β2AR-green fluorescent protein chimeras (β2AR-GFP) were shown to localize in compartments that accumulated fluorescently-labeled dextran. However, the exact nature of these compartments was not defined and the intracellular itineraries of β2ARs during downregulation were not fully examined. In the present study, we determine the intracellular events leading to agonist-induced β2AR downregulation and specifically demonstrate the sorting of receptors to lysosomes during prolonged exposures to agonist.

MATERIALS AND METHODS

Materials

12β6 cells (a gift of B. Kobilka, Stanford, CA) were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum and 200 μg/ml Genetecin (Life Technologies, Gaithersburg, MD). This cell line was derived by stable transection of HEK293 cells, and expresses human β2ARs with an N-terminal epitope tag derived from influenza hemagglutinin (HA-tag) at a level of 300,000/cell (Moore et al., 1995; von Zastrow and Kobilka, 1992). Mouse monoclonal antibodies against the HA-tag were 12C5 (obtained from Boehringer-Mannheim, Indianapolis, IN) or mHA.11 (from Berkeley Antibody Co., Berkeley, CA). A mouse monoclonal antibody against human transferrin receptors was purchased from Pharmingen (San Diego, CA), and rabbit polyclonal antiserum against human cathepsin D and rab11 were obtained from Calbiochem (La Jolla, CA) and Zymed Laboratories (South San Franciscio, CA), respectively. Species-appropriate goat secondary antibodies were purchased from Molecular Probes (Eugene, OR). [3H]CGP12177 (44 Ci/mmol) was obtained from Dupont-New England Nuclear (Boston, MA). Texas Red conjugated bovine serum albumin (BBA-Texas Red) was purchased from Molecular Probes, and digitonin from Boehringer-Manheim. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Measurement of receptor downregulation

12β6 cells were grown in poly-L-lysine coated 12-well clusters to 50-70% confluence, then exposed to 5 μM isoproterenol (ISO) in triplicate wells at 37°C for 24 hours or left untreated. Some wells were treated with 8-bromo-cAMP (1 mM) alone or in addition to ISO. In the protease inhibitor experiments, cells were treated similarly with ISO except that leupeptin (100 μM) and/or pepstatin (100 μM) were added one hour prior to and 12 hours after the addition of ISO. Control cells received the same concentrations of vehicle alone (0.1% dimethyl sulfoxide, Me2SO). Following agonist treatment, the cells were washed four times with ice-cold phosphate buffered saline (PBS), then incubated with 6 nM [3H]CGP12177 in DMEM with 20 mM Hepes, pH 7.4 (DMEM-H) containing 0.05% digitonin at 4°C for 90 minutes. The cells were washed twice with DMEM-H, then lysed in the wells with 0.1% Triton X-100, and the lysates counted by scintillation spectroscopy. Nonspecific binding of radioligand was determined by parallel incubations in the presence of propranolol (3 μM), and was less than 5% of total. Statistical significance was determined using a one-way ANOVA and defined by P<0.05.

Previous control experiments to evaluate the need for inhibitors of oxidation demonstrated that the extent of ISO-induced β2AR downregulation was not altered upon the addition of ascorbic acid (0.1 mM) and thiourea (1 mM). Also, following a 24 hour incubation with 12β6 cells, medium containing ISO alone was able to fully stimulate receptor internalization.

Measurement of β2AR internalization and recycling kinetics

12β6 cells were grown to 50-70% confluence in poly-L-lysine-coated 24-well clusters and pretreated for 6 hours with leupeptin (100 μM) and pepstatin (100 μM) or left untreated as a control. Cells were exposed to ISO (5 μM) in triplicate wells for varying times up to 20 minutes, washed with ice-cold DMEM-H, and incubated with 6 nM [3H]CGP12177 in DMEM-H at 4°C for 90 minutes in the absence of digitonin to measure surface receptors only. The cells were washed, lysed and counted as described above. Nonspecific binding of radioligand was determined by parallel incubations in the presence of 3 μM propranolol, and was less than 5% of total. The fraction of receptors left on the cell surface was plotted versus time of agonist exposure, and the curves fitted by nonlinear regression using the computer program GraphPad Prism (version 2; San Diego, CA). The rate of approach to a steady-state between surface and internal receptors is determined by first-order rate constants for receptor endocytosis (k0) and recycling (k0). Unique values for these rate constants were estimated by curve-fitting to equation 4 of Morrison et al. (1996), and the level of statistical significance was determined using a paired Student’s t-test and defined by P<0.05. Rate constants are presented as the mean ± s.e.m. of five independent experiments.

In separate experiments, 12β6 cells were pretreated with leupeptin (100 μM) in the presence and absence of bafilomycin A1 (1 μM) for 30 minutes, then exposed to ISO (5 μM) for 6 hours. Cells were harvested by tituration in ice-cold DMEM-H and washed by centrifugation. To allow receptor recycling, cells were suspended in DMEM-H prewarmed to 37°C and containing 10% normal goat serum and 6 nM [3H]CGP12177. Samples containing 75 μg of protein were removed at varying times up to 60 minutes, rapidly diluted into ice-
cold DMEM-H containing 6 nM [3H]CGP12177, and left on ice for 60 minutes for binding to equilibrate. The cells were collected by rapid filtration and the bound tritium quantified by scintillation spectroscopy. Nonspecific binding of radioligand was determined by parallel incubations with 3 μM propranolol and was less than 5% of total binding. The fraction of receptors on the cell surface was plotted versus time of recycling, and the curves fitted by nonlinear regression as described by Morrison et al. (1996). Recycling rate constants are presented as the mean ± s.e.m. of three separate experiments.

**Immunofluorescence laser confocal microscopy**

12β6 cells were grown on poly-L-lysine-coated 22 mm glass coverslips to 50-70% confluence in 6-well clusters and treated as indicated in the figure legends. In some experiments, prior to ISO treatment, the cells were fed BSA-Texas Red (5 mg/ml) in DMEM-H for one hour at 37°C, washed twice with DMEM-H, and chased for one hour at 37°C with DMEM-H to label lysosomes. In separate experiments, cells were pretreated with 1 μM bafilomycin A1 in MezSO (final concentration, 0.1%) for 30 minutes prior to the addition of agonist. In control experiments, cells were pretreated with 0.1% MezSO alone. Following treatment, the cells were washed with PBS containing 1.2% sucrose (PBSS), fixed with 4% paraformaldehyde in PBSS at 4°C for 10 minutes, then washed again with PBSS. The following steps were done at room temperature, with PBSS used for washes. The fixed cells were incubated in 0.34% L-lysine, 0.05% Na-mperiodate for 20 minutes, then washed and permeabilized with 0.2% Triton X-100. After further wash, the cells were blocked with 10% heat-inactivated goat serum (HIGS) for 15 minutes. Primary antibodies were diluted in PBSS with 0.2% HIGS and 0.05% Triton X-100, then added to the cells and left for 1-12 hours. The cells were washed four times before labeling with secondary antibodies using the same procedure as for the primary antibodies. We used the following concentrations of antibodies: 12CA5, 1 μg/100 μl; mHA.11, 0.5 μg/100 μl; monoclonal anti-transferrin receptor, 2 μg/100 μl; anti-cathepsin D, 2 μl/100 μl; anti-rab11, 1 μg/100 μl; FITC-anti-mouse IgG, 1:100 dilution; Texas Red anti-rabbit IgG, 1:200 dilution. The coverslips were mounted in Mowiol and viewed using a Molecular Dynamics Multiprobe 2001 laser confocal scanning microscope. Optical sections through cells were collected at a thickness of 0.49 μm. The digitalized images were transferred to Adobe Photoshop for processing, and printed using a Codonics 1600 dye sublimation printer.

**RESULTS**

We determined the extent of β2AR downregulation in HEK293 cells stably expressing a human β2AR with an N-terminal HA-epitope tag (12β6 cells) (von Zastrow and Kobilka, 1992). Total binding sites (surface and internal) for the βAR-antagonist [3H]CGP12177 were measured in digitonin-permeabilized cells before and after chronic agonist exposure (Fig. 1). Using this method, we found that approximately 30% of total cellular receptors were no longer detectable (i.e. downregulated) following 24 hours of treatment with 5 μM ISO. There were no changes in cell numbers, total protein, or cell viability (Trypan Blue exclusion) after a 24 hour ISO treatment compared with untreated control cells, excluding altered growth or viability as a cause of agonist-induced loss of radioligand binding sites (data not shown). Since cAMP-dependent mechanisms also have been shown to induce β2AR loss in some cell systems, we determined the extent of receptor downregulation in 12β6 cells treated with 1 mM 8-bromo cAMP in the absence and presence of agonist. While cAMP alone significantly reduced receptor levels by approximately 13%, this effect was additive to that seen with agonist, suggesting different mechanisms for cAMP- and agonist-induced β2AR downregulation. To determine if lysosomal proteases contributed to the observed agonist-induced loss of receptors, we measured agonist-induced downregulation in cells pretreated with the protease inhibitors leupeptin and pepstatin, which have been shown previously to block ligand-activated degradation of epidermal growth factor receptors in rat hepatocytes (Renfrew and Hubbard, 1991). In 12β6 cells, pretreatment with these protease inhibitors almost completely blocked agonist-induced β2AR downregulation (Fig. 2).
Since inhibiting lysosomal proteases significantly blocked β2AR downregulation, it seemed likely that downregulation might require the trafficking of receptors to lysosomes during prolonged agonist exposure. To determine whether this might be the case, we tracked receptors using immunofluorescence laser confocal microscopy. To label lysosomes, we used two markers: antibodies against cathepsin D, an aspartic lysosomal protease (Tang and Wong, 1987) which also localizes with the lysosomal integral membrane protein LAMP-1 in 12ß6 cells (data not shown); and BSA-Texas Red, a fluorescent fluid-phase marker given to cells in a pulse-chase manner to label lysosomes (Racoosin and Swanson, 1994). We treated 12ß6 cells with ISO in the presence and absence of leupeptin and pepstatin for varying times up to 6 hours and examined specimens for the localization of HA-tagged β2ARs with either cathepsin D or BSA-Texas Red. In protease inhibitor-treated cells, there was minimal localization of β2ARs with cathepsin D (Fig. 3A-C) or BSA-Texas Red (data not shown) following a 5 minute exposure to agonist. Very little colocalization also was observed after treatment periods up to 60 minutes (data not shown). However, after 6 hours of agonist treatment, increased localization of β2ARs with both cathepsin D (Fig. 3D-F) and BSA-Texas Red (Fig. 3G-I) was observed. Significant localization also was observed after 3 hours of agonist treatment (data not shown). Some intracellular β2ARs do not localize with cathepsin D but substantially localize with transferrin receptors (data not shown), and most likely represent β2ARs that have not yet reached lysosomes or have been sorted from endosomes for transit to the plasma membrane. No internalized receptors were seen following 24 hours of exposure to 8-bromo cAMP alone (data not shown).

In the absence of protease inhibitors, there was little or no localization of receptors with either cathepsin D or BSA-Texas Red, even after 6 hours of ISO exposure (Fig. 4). Instead, these receptors almost completely overlapped with transferrin receptors (data not shown). Although this result was likely a result of degradation of the β2AR N-terminal HA epitope by lysosomal proteases, we also considered the possibility that treatment with leupeptin and pepstatin may alter vesicular trafficking by inducing a nonspecific sorting of receptors to lysosomes. To test for this possibility, we first determined if transferrin receptors localized with cathepsin D in cells incubated with β-agonist in the presence and absence of protease inhibitors. Normally, transferrin receptors undergo constitutive internalization from the cell surface into early endosomes and the perinuclear recycling compartment and then recycle to the plasma membrane, with few receptors ever reaching lysosomes (Daro et al., 1996; Dautry-Varsat et al.,...
We found minimal localization of transferrin receptor with cathepsin D in either control or in protease inhibitor-treated cells, suggesting that leupeptin and pepstatin did not cause a nonspecific shunting of endosomal receptors to lysosomes (data not shown). In addition, if normal endosomal sorting of receptors were affected by the pretreatment of cells with leupeptin and pepstatin, we considered it likely that the first-order rate constants of agonist-induced β2AR endocytosis \( (k_e) \) and recycling \( (k_r) \) would also be altered. However, the rate constants for agonist-induced β2AR endocytosis during a 20 minute exposure to ISO were not significantly different comparing control cells and cells pretreated with protease inhibitors for 6 hours \( (k_e \text{ for control cells} = 0.171\pm0.014 \text{ minute}^{-1}; k_e \text{ for protease inhibitor treated cells} = 0.151\pm0.024 \text{ minute}^{-1}) \). In addition, values for \( k_r \), derived from curve-fitting as previously described (Morrison et al., 1996), did not differ significantly in the two groups \( (k_r \text{ for control cells} = 0.132\pm0.020 \text{ minute}^{-1}; k_r \text{ for protease inhibitor treated cells} = 0.135\pm0.041 \text{ minute}^{-1}) \).

We next performed studies to determine which class of lysosomal protease is responsible for β2AR downregulation. Cells were incubated with either leupeptin (100 μM), which inhibits cysteine proteases such as cathepsins B, H, and L, or pepstatin (100 μM), which inhibits the activity of the aspartic protease, cathepsin D (Erickson, 1989). As shown in Fig. 2, most of the receptor downregulation following a 24 hour exposure to agonist was inhibited by leupeptin, while pepstatin had no effect. This result is supported by morphologic data indicating that the localization of HA-β2AR with cathepsin D is maintained in the presence of leupeptin alone (Fig. 5A-C) and is lost when pepstatin is used alone (Fig. 5D-F). The effect of leupeptin might be explained if it permitted the accumulation of β2ARs in lysosomes as a result of normal receptor turnover. If this were the case, we should observe localization of β2ARs with cathepsin D during prolonged leupeptin exposure even in the absence of agonist. To test for this, 12β6 cells were incubated with leupeptin alone for 30 hours, the maximal estimated half-life of β2ARs in untreated cells (Mahan and Insel, 1986), then fixed and labeled with antibodies to localize both β2ARs and cathepsin D. In examining 300 cells treated with leupeptin, only 6 cells appeared to have a small amount of punctate staining for β2ARs, a fraction similar to that observed in 12β6 cells not treated with leupeptin, and no receptors localized with cathepsin D (data not shown).

We and others (Moore et al., 1995; von Zastrow and Kobilka, 1992) have shown that β2ARs enter early endosomes following brief exposures to agonist. To determine if β2ARs transit the normal endocytic pathway en route to degradation within lysosomes during chronic exposures to agonist or use some alternative pathway, we studied the effects of bafilomycin A1, a vacuolar-type H⁺-ATPase inhibitor that raises endosome and lysosome pH (Yoshimori et al., 1991) and blocks the formation of vesicles carrying endocytosed materials from early endosomes to lysosomes (Clague et al., 1994; van Deurs et al., 1996). In cells pretreated with bafilomycin A1 (1 μM) in the presence of leupeptin followed by ISO for 6 hours, little or no colocalization of β2ARs and cathepsin D was observed (Fig. 6A-C), whereas significant colocalization of the two markers was noted in cells not pretreated with bafilomycin A1 (compare with Fig. 5A-C). Indeed, in most cells, receptors appeared in a dense perinuclear compartment which was completely devoid of cathepsin D labeling. To determine the nature of this compartment, we labeled cells treated similarly in the presence of bafilomycin A1.
and absence of bafilomycin A₁ with antiserum against the perinuclear recycling compartment marker, rab11 (Daro et al., 1996; Green et al., 1997). In cells exposed to ISO for 6 hours in the presence of leupeptin alone, there was only a small degree of $\beta_2$AR localization with rab11 (Fig. 6G-I), which increased with more prolonged exposures up to 18 hours (data not shown). However, pretreatment with bafilomycin A₁ caused the marked accumulation of $\beta_2$ARs in a rab11-containing perinuclear compartment (Fig. 6D-F) and significantly slowed the rate of receptor recycling to the plasma membrane ($k_r$ following 6 hour exposure to ISO = 0.076±0.014; $k_r$ for cells pretreated with bafilomycin A₁ = 0.024±0.007) without affecting the final extent of recycling at 60 minutes. Despite this striking redistribution of $\beta_2$ARs, bafilomycin A₁ did not noticeably alter the distribution of transferrin receptors (data not shown), similar to previously published data (Johnson et al., 1993).

**DISCUSSION**

The data reported here provide direct evidence that the agonist-dependent loss of $\beta_2$ARs defined by radioligand binding is caused by the specific trafficking of these receptors to lysosomes for degradation. Previous studies of $\beta_2$AR downregulation have used the loss of radioligand binding as an index of receptor degradation, an activity which in principle may be affected by factors other than receptor degradation, including a selective inhibition of receptor synthesis. In addition to demonstrating an important role for proteolysis in $\beta_2$AR downregulation, we also define the cellular basis for downregulation by showing that the mechanism of $\beta_2$AR downregulation involves the specific, agonist-induced sorting of receptors from endosomes to lysosomes.

This interpretation of our immunofluorescence data depends upon the identification of lysosomes in 12β6 cells, and for this purpose, we used three criteria: (1) the accumulation of BSA-Texas Red with long labeling and chase times (Fig. 2); (2) labeling with an antibody against human cathepsin D (Fig. 2); (3) labeling with an antibody against LAMP-1, in a pattern overlapping with that of cathepsin D (data not shown). Although proteases such as cathepsin D can be found within endosomes in other cell types, possibly during protease maturation and transport to lysosomes, the abundance of such proteases is much lower in endosomes compared with lysosomes (Rodman et al., 1990).

The inclusion of protease inhibitors in the incubation medium was necessary for the immunofluorescence localization of receptors in lysosomes (Fig. 4), most likely because the HA-epitope tag on the receptor N terminus would otherwise be degraded in protease-containing compartments. To exclude nonspecific effects of leupeptin, we examined

![Fig. 5. Specificity of lysosomal proteases in $\beta_2$AR localization with cathepsin D. Cells were treated as in Fig. 2 except that leupeptin or pepstatin (100 μM each) were added individually. Localization of HA-$\beta_2$ARs with cathepsin D is observed in leupeptin-treated (A-C) but not in pepstatin-treated (D-F) cells. Bar, 10 μm.](image-url)
several parameters of intracellular receptor trafficking events. Leupeptin had no detectable effect on the intracellular distribution of transferrin receptors relative to lysosomes in the presence of agonist, because under these conditions, there was no localization of transferrin receptors with cathepsin D (data not shown). This experiment also shows that the effect of agonist is receptor-specific, because under the same conditions, β2ARs were extensively localized with this lysosome marker (Fig. 3). This result is of further significance since transferrin receptors and β2ARs both traffic through early endosomes (Moore et al., 1995; von Zastrow and Kobilka, 1992), and indicates that prolonged agonist treatment results in the specific sorting of β2ARs, but not transferrin receptors, from endosomes to lysosomes. Protease inhibitors were not nonspecifically affecting normal endosomal trafficking of β2ARs, because the incubation of cells with leupeptin and pepstatin for 6 hours had no affect upon the rates of β2AR endocytosis and recycling over a subsequent short time course of agonist exposure. Incubation of 12β6 cells in the presence of leupeptin by itself did not cause localization of β2ARs with cathepsin D (data not shown), indicating that the accumulation of these receptors in lysosomes required prolonged treatment with agonist. Moreover, in the absence of agonist and the presence of leupeptin, there was no significant change in the receptor number as assessed by radioligand binding, suggesting that protease inhibitor was not influencing the basal half-life of β2ARs (Fig. 2). These data strongly suggest that the localization of β2ARs with lysosomal markers we observed was not the result of receptor trafficking in association with normal receptor turnover and was instead dependent on agonist.

The finding that the protease inhibitor leupeptin was needed to reveal β2AR trafficking to lysosomes and at the same time blocked receptor downregulation is strong evidence that these processes are mechanistically related. An apparent temporal disconnection between the occurrence of measurable downregulation (24 hours of agonist exposure), and trafficking to lysosomes (3-6 hours), can be explained by the fact that ligand binding is relatively resistant to proteases and does not require the N-terminal domain (Rands et al., 1990). Also, since the ligand binding pocket is sequestered within the lipid bilayer (Liggett, 1997), radioligand-binding activity might be expected to persist in the presence of proteases for a longer period of time than an epitope tag exposed to the interior of lysosomes. Further, although trafficking to lysosomes is observed within 3-6 hours (Figs 3 and 5A-C), the quantity of receptor providing the immunofluorescence signal could be relatively small compared with the total receptor population. Although proteolysis of the HA epitope on the receptor’s N terminus is not necessarily synonymous with degradation of its ligand binding site, these data suggest that lysosomal cysteine proteases are largely responsible for the downregulation of

**Fig. 6.** Inhibition of β2AR traffic to lysosomes by bafilomycin A1. Cells were pretreated with leupeptin (100 μM) for 30 minutes in the presence (A-F) or absence (G-I) of bafilomycin A1 (1 μM) before adding ISO (5 μM) for 6 hours. Cells were labeled with mHA.11 (B,E,H) and antisera against either cathepsin D (A) or rab11 (D and G). Merged images are shown in C, F, and I. Minimal localization of HA-β2ARs with cathepsin D is noted in A-C. There is a striking redistribution of β2ARs to a rab11-containing perinuclear compartment in cells pretreated with bafilomycin A1 (D-F) compared to those treated with ISO and leupeptin alone (G-I). Bar, 10 μm.
HA-β2ARs. Further, although β2ARs localize with cathepsin D, this aspartic protease appears to contribute minimally, if at all, to its downregulation.

The finding that ISO-induced downregulation was almost completely inhibited by the addition of protease inhibitor, together with the small increment of 8-bromo cAMP-induced downregulation, suggests that receptor degradation is the major component of agonist-induced downregulation in 12β6 cells. Further, since transcription in the 12β6 cell line is under the control of a viral promoter element (Cullen, 1987) whose activity is not likely to be affected by agonist treatment, our measurements probably underestimate the true extent of receptor degradation since there is likely to be some continued receptor synthesis during the 24 hours of agonist treatment. This would not confound the interpretation of our trafficking data, since newly synthesized receptors are rapidly transported to the cell surface, where they bind agonist and begin their continuous rounds of endocytosis and recycling (Morrison et al., 1996). Although the 3′ untranslated region of β2AR cDNA expressed in this cell line has all four cAMP-dependent mRNA destabilization motifs (Danner et al., 1998; von Zastrow and Kobilka, 1992), the effect of 8-bromo cAMP on receptor downregulation was small and additive to that seen with ISO alone (Fig. 1). Further, if the small amount of 8-bromo cAMP-mediated β2AR downregulation that we measured were due to degradation in lysosomes, we would have expected there to be localization of receptors with cathepsin D, because such colocalization was observed in agonist treated cells at times points (3 hours) before significant agonist-induced downregulation was detected (data not shown). These results suggest that the mechanisms of receptor downregulation mediated by cAMP and by agonist are separable and that cAMP-dependent events contributed little to agonist-induced β2AR downregulation in 12β6 cells, similar to results from studies using BEAS-2B cells (Kelsen et al., 1997) and signal transduction defective S49 lymphoma (cyt- and kin-) cell lines (Proll et al., 1992).

Other studies have examined the half-life of β2AR radioligand binding activity in the presence and absence of agonist. Estimates were made by the use of an irreversible antagonist and computer modeling of receptor synthesis and degradation (Mahan and Insel, 1986; Nantel et al., 1994; Neve and Molinoff, 1986). All of these studies used loss of radioligand binding activity as an index of receptor degradation, and none were able to address the molecular or cellular mechanisms of downregulation. Several other studies have evaluated the trafficking of β2ARs to lysosomes. β2ARs in A431 cells pre-bound with gold-labeled monoclonal anti-receptor antibody apparently traffic to lysosomes; however, this process is not agonist-dependent, was not reported to correlate with downregulation, and initiates through non-clathrin coated vesicles (Raposo et al., 1992). In contrast, agonist-induced receptor endocytosis in the 12β6 line used in this study occurs through clathrin-coated vesicles (Moore et al., 1995; von Zastrow and Kobilka, 1994), suggesting that the receptor trafficking events we observed are specific and not related to bulk turnover of membrane proteins. In other recent reports prolonged exposures to agonist caused the apparent trafficking of a β2AR-green fluorescent protein chimera (β2AR-GFP) to a rhodamine-dextran-labeled compartment, presumably lysosomes, in stably transfected HeLa cells and transiently transfected HEK293 cells (Gagnon et al., 1998; Kallal et al., 1998). Interpretation of these results is complicated by the presence of a significant pool of intracellular β2ARs-GFP in untreated cells, suggesting abnormal processing of this protein within the endoplasmic reticulum and constitutive trafficking of receptors to undefined intracellular compartments. The cells used in the present study express a β2AR with a small N-terminal epitope tag, and no intracellular receptors can be identified in the absence of agonists (data not shown; Moore et al., 1995; von Zastrow and Kobilka, 1992). Agonist-dependent downregulation of these receptors correlates with their intracellular trafficking to protease-containing compartments, which are identified by several criteria as lysosomes (Fig. 3). The colocalization of β2ARs-GFP with rhodamine-dextran did not require the presence of protease inhibitors (Gagnon et al., 1998; Kallal et al., 1998), suggesting that either the C terminus of β2ARs-GFP is relatively resistant to proteolysis or that these receptors localized with rhodamine-dextran in a compartment devoid of proteases. Our data clearly show proteolytic action on receptors, as assessed by loss of their N-terminal HA-epitopes and that inhibition of proteases also inhibits downregulation.

A role for clathrin-mediated receptor endocytosis in the process of downregulation has been suggested in a recent study in which a dominant negative mutant of dynamin (K44A) almost completely abolished agonist-induced β2AR downregulation in HEK293 cells, although the effects were less in HeLa and COS cells (Gagnon et al., 1998). However, it was unclear if β2ARs that are destined for degradation in lysosomes traffic through endosomes or use an alternate pathway. In the present study, treatment with bafilomycin A1, which prevents acidification of endosomes and lysosomes in many cell systems, including HEK293 cells (Shrader-Fischer and Paganetti, 1996; Yoshimori et al., 1991), significantly inhibited the localization of β2ARs with cathepsin D (Fig. 6A-C), caused the enrichment of receptors in the rab11-containing perinuclear recycling compartment (Fig. 6D-F), and slowed the rate of β2AR recycling following a 6 hour exposure to agonist. Bafilomycin A1 is known to inhibit the formation of endosomal carrier vesicles between early and late endosomes (Clague et al., 1994), to modestly slow the externalization of transferrin receptors from the perinuclear recycling compartment (Johnson et al., 1993; Presley et al., 1997), and to prevent the transport of horseradish peroxidase (van Weert et al., 1995) and cationized gold (van Deurs et al., 1996) to lysosomes. Our data indicate that the transport to lysosomes of β2ARs destined for degradation also is dependent on a functional vacuolar-type H+-ATPase and provide strong evidence that β2ARs are specifically sorted from endosomes en route to degradation in lysosomes by a pH-dependent mechanism. A bafilomycin A1-sensitive mechanism has been described in the recycling of transferrin receptors to the plasma membrane (Johnson et al., 1993; Presley et al., 1997), a process which requires the presence of a critical YTRF motif within the receptor’s cytoplasmic tail. However, a bafilomycin A1-sensitive mechanism previously has not been implicated in the trafficking of any G protein-coupled receptors from endosomes to lysosomes or to any other intracellular destination. Further, the accumulation of β2ARs in the perinuclear recycling compartment observed in bafilomycin A1-treated cells can probably be explained by both a decreased efflux of receptors...
from this compartment (Johnson et al., 1993; Presley et al., 1997) and an increased influx due to the diversion of receptors from lysosomes, although an additional bafilomycin A1-sensitive pathway from the perinuclear recycling compartment to lysosomes cannot be excluded.

The accumulated data thus indicate that in HEK293 cells β2ARs are dynamically sorted from early endosomes to at least three destinations, directly to the plasma membrane by rapid recycling; the perinuclear recycling compartment, from which slow recycling to the cell surface occurs; and lysosomes, where they are degraded by cysteine proteases. Most previous studies where the trafficking of other GPCRs to lysosomes has been demonstrated have been restricted to receptors that recycled to only a small extent. Activated thrombin receptors quite rapidly localize with LAMP-1, with a limited degree of receptor recycling (Hoxie et al., 1993). LH/hCG receptors labeled with immunogold move rapidly into lysosomes and multivesicular bodies (possibly late endosomes), and little or no recycling of label or receptors is observed (Ghinea et al., 1992). Because GPCRs predominantly targeted to lysosomes and those that are largely recycled, such as β2ARs, traffic through similar endocytic pathways (Hein et al., 1994), it is interesting to speculate on the presence of specific sorting signals within their C-terminal domains. Candidate signals include dileucine or tyrosine-based motifs (for review see Trowbridge et al., 1993; Bonifacino et al., 1996), which may promote receptor interactions with adaptor proteins such as AP-3 (Dell’Angelica et al., 1997) or sorting nexins (Kurten et al., 1996). Additional studies are underway to define the complex relationships among receptor endocytosis, sorting to lysosomes, and receptor recycling during prolonged agonist exposure.

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REFERENCES


Morrison, K. J., Moore, R. H., Carsrud, N. D. V., Millman, E. E., Trial, β2-adrenergic receptors and lysosomes 337


