Role of Clathrin-mediated Endocytosis in Agonist-induced Down-regulation of the β2-Adrenergic Receptor*

(Received for publication, December 3, 1997, and in revised form, January 20, 1998)

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Previous studies have demonstrated that non-visual arrestins function as adaptors in clathrin-mediated endocytosis to promote agonist-induced internalization of the β2-adrenergic receptor (β2AR). Here, we characterized the effects of arrestins and other modulators of clathrin-mediated endocytosis on down-regulation of the β2AR. In COS-1 and HeLa cells, non-visual arrestins promote agonist-induced internalization and down-regulation of the β2AR, whereas dynamin-K44A, a dominant-negative mutant of dynamin that inhibits clathrin-mediated endocytosis, attenuates β2AR internalization and down-regulation. In HEK293 cells, dynamin-K44A profoundly inhibits agonist-induced internalization and down-regulation of the β2AR, suggesting that receptor internalization is critical for down-regulation in these cells. Moreover, a dominant-negative mutant of β-arrestin, β-arrestin-(319–418), also inhibits both agonist-induced receptor internalization and down-regulation. Immunofluorescence microscopy analysis reveals that the β2AR is trafficked to lysosomes in HEK293 cells, where presumably degradation of the receptor occurs. These studies demonstrate that down-regulation of the β2AR is in part due to trafficking of the β2AR via the clathrin-coated pit endosomal pathway to lysosomes.

Stimulation of G protein-coupled receptors (GPRs) leads to adaptive changes that serve to modulate the responsiveness of a cell to further stimulation (1). Three temporally distinct mechanisms of agonist-promoted regulation of GPRs have been identified: desensitization, internalization, and down-regulation. Desensitization often occurs within seconds of stimulation and for many GPRs is mediated by phosphorylation of the receptor by a specific G protein-coupled receptor kinase. This phosphorylation promotes the binding of an arrestin to the receptor, which sterically prevents subsequent coupling to G proteins. Agonist stimulation also promotes rapid (minutes) internalization of many GPRs into an intracellular endosomal pool, thereby effectively removing the receptor from the cell surface. Finally, prolonged receptor stimulation (hours) results in receptor down-regulation, defined by an overall decrease in receptor number.

The β2AR-adrenergic receptor (β2AR) has served as an important model for elucidating the molecular mechanisms involved in GPR regulation. The β2AR is rapidly desensitized after agonist stimulation, in part a consequence of phosphorylation of carboxyl-terminal serines and threonines by G protein-coupled receptor kinase 2 (2). This phosphorylation promotes the binding of an arrestin (either β-arrestin or arrestin 3), which directly inhibits the ability of the receptor to couple to Gs (3–5). Recent studies have suggested that β-arrestin and arrestin 3 are not only involved in the initial stages of desensitization of the β2AR but that they are important components in receptor internalization (6, 7). Since both β-arrestin and arrestin 3 can specifically bind to clathrin, the major structural protein of clathrin-coated pits, arrestins can function as adaptors to recruit G protein-coupled receptor kinase-phosphorylated receptors into coated pits where they can be internalized (7). Recent studies have demonstrated that β2AR internalization plays an important role in receptor resensitization, a process that involves dephosphorylation of the receptor and subsequent recycling back to the cell surface (8–10).

It has been hypothesized that trafficking of GPRs from early endosomes to lysosomes might be a mechanism for agonist-induced down-regulation. Indeed several GPRs, including the thrombin (11), thyrotropin (12), and cholecystokinin (13) receptors, have been shown to be sorted to lysosomes in an agonist-dependent manner. We have also recently shown that a β2AR tagged with the green fluorescent protein (GFP) can also slowly accumulate in lysosomes after agonist activation (14). However, whether internalization of the β2AR via clathrin-coated pits directly contributes to its down-regulation remains speculative. Previous radioligand binding studies utilizing mutants of the β2AR have failed to link internalization and down-regulation and, in fact, suggest that the two processes may be distinct events (15–19). Moreover, in addition to protein degradation, prolonged agonist stimulation also leads to decreased steady state levels of β2AR mRNA in some cells (20–22). Thus, down-regulation of the β2AR is composed of multiple mechanisms that function to both destroy existing receptor as well as decrease the synthesis of new receptors.

The present studies were focused on addressing whether agonist-promoted internalization of the β2AR contributes to its down-regulation. To accomplish this, we expressed the β2AR in a variety of mammalian cell lines and examined the effect of coexpression of various modulators of clathrin-mediated endocytosis on agonist-induced down-regulation. The results of these studies indicate that increases or decreases in agonist-mediated internalization of the β2AR directly correlate with concomitant changes in the extent of receptor down-regulation. Moreover, in HEK293 cells, β2AR down-regulation appears...
very dependent upon internalization of the receptor and subsequent trafficking to lysosomes.

EXPERIMENTAL PROCEDURES

**Materials—**A BamHI fragment of dynamin-K44A was subcloned into BamHI-digested pcDNA3, whereas Flag-tagged β2AR was subcloned into pcDNA3 as described (14). Poly-L-lysine was obtained from Sigma, and tissue culture dishes were treated according to manufacturer’s instructions. The construct pcDNA3-β2AR-GFP, encoding a β2AR fusion protein with GFP at its carboxyl terminus, was created as described (14). Flag-β2AR and arrestin constructs in the vector pBC12BI have been described (7).

**Cell Culture and Transient Transfection—**COS-1, HEK293, and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (complete media) at 37 °C in a humidified atmosphere of 85% air, 5% CO₂. Cells grown to 80–90% confluency in T75 flasks were transfected with 65 μl (COS-1 and HeLa) or 90 μl (HEK293) of LipofectAMINE (Life Technologies) according to the manufacturer’s instructions. For transfection of COS-1 cells, 10 μg of pBC-Flag-β2AR was transfected alone or cotransfected with 3 μg of pBC-β-arrestin, pBC-arrestin 3, or pBC-arrestin. HeLa cells and HEK293 cells were transfected with 5–10 μg of pcDNA3-Flag-β2AR or cotransfected with 3 μg of pBC-lo or 5 μg (HEK293) of pcDNA3-arrestin 3, pcDNA3-β-arrestin, or pcDNA3-dynamin-K44A. For microscopy studies, HEK293 cells were transfected as above with 10 μg of pcDNA3-β2AR-GFP.

**Sequestration Assays—**For COS-1 and HeLa cells, receptor internalization studies were performed as described (7). Briefly, cells were harvested 48 h after transfection by trypsinization, washed extensively, resuspended, and split into 0.5 ml aliquots in phosphate-buffered saline (PBS) containing 0.1 mM ascorbate. Cells were incubated with or without 1 μM (-)-isoproterenol for 37 °C for the indicated time, washed with ice-cold PBS, and resuspended in PBS for binding studies. Cell surface receptors were measured by incubating with 10 nM [3H]CGP-12177 for 1 h at 14 °C with or without 10 μM (-)-alprenolol. Protein concentrations were determined by Bio-Rad assay using bovine serum albumin as a standard.

For HEK293 cells, cells were trypsinized 24 h after transfection and plated onto poly-L-lysine-coated 100-mm tissue culture dishes and incubated overnight. The cells were then washed with PBS, and 10 ml of Dulbecco’s modified Eagle’s medium containing 0.1 mM ascorbate was added to each dish. The cells were equilibrated to 37 °C and incubated for the indicated time with or without 1 μM (-)-isoproterenol. The medium was replaced with 2 ml of 0.05% trypsin and, after 1–2 min at 37 °C, trypsinization was stopped with 10 ml of complete media and dilution to 50 ml with ice-cold PBS. Cells were harvested by centrifugation, washed once in 50 ml ice-cold PBS, and resuspended in 2–2.5 ml of PBS. Cell surface receptors were measured using [3H]CGP-12177 for 3 h at 14 °C with or without 10 μM (-)-alprenolol. Protein concentrations were determined by Bio-Rad assay using bovine serum albumin as a standard.

**Down-regulation Assays—**Cells were harvested by trypsinization 24 h after transfection and plated onto 60-mm dishes (poly-L-lysine treated for HEK293). The next day, complete media containing 0.1 mM ascorbate was added, and the cells were then treated with or without 10 μM (-) isoproterenol for 1 to 24 h. Cells were washed four times with 4 ml of room temperature PBS and then lysed in 1–4 ml of 20 mM Tris, pH 8, 2 mM EGTA, 5 mM EDTA, 1.5 μg/ml leupeptin (–)/0.2 μg/ml benzamidine by homogenization on ice using a polytron (25,000 rpm, 2 x 15 s). Receptor expression was measured in the cell lysate with 1 ml [3H]lido-

dopindolol (NEN Life Science Products) at room temperature for 1 h with or without 10 μM (-)-alprenolol. Binding reactions were terminated by the addition of 5 × 4 ml of ice-cold 25 mM Tris, pH 7.5, 2 mM MgCl₂, followed by rapid filtration through Whatman GF/C filters using a Brandel Cell Harvester.

**Immunofluorescence Microscopy—**HEK293 cells were transiently transfected with pcDNA3-β2AR-GFP using LipofectAMINE according to the manufacturer’s instructions. The cells were trypsinized and plated onto poly-L-lysine-coated coverslips 24 h after transfection. After the cells had adhered, the media was aspirated and replaced with 1 ml of complete media plus 0.1 mM ascorbate. For labeling of lysosomes, 100 μM dihydrochloride (NBD)-labeled dextran (Molecular Probes) was added to each well and allowed to incubate at 37 °C overnight. The dextran was washed out 1.5 h before the end of the incubation period and replaced with fresh media. Isoproterenol (10 μM final) was added to the media or rhodamine-dextran mix at the appropriate time. At the end of the incubation, cells were washed briefly three times in PBS, fixed for 10 min in 3.7% formaldehyde, and mounted on coverslips using SlowFade mounting medium (Molecular Probes). Confocal microscopy analysis was performed on a Bio-Rad MRC-Zeiss Axiovert 100 confocal microscope (Hemmelholstade) using a Zeiss Plan-Apo 63× 1.4 NA oil immersion objective.

**RESULTS AND DISCUSSION**

Since mutational analysis has not provided a clear picture of the link between agonist-stimulated internalization and down-regulation of the β2AR, we undertook studies examining the effect of modulators of endocytosis on agonist-mediated down-regulation of the wild-type β2AR. Presumably if internalized receptors are targeted for down-regulation, increasing or decreasing the proportion of these receptors should have a concomitant effect on down-regulation. Previously we demonstrated that non-visual arrestins increase agonist-induced internalization of the β2AR in COS-1 cells (7). Thus, COS-1 cells provide a convenient model for establishing a potential link between agonist-mediated internalization and down-regulation of the β2AR. COS-1 cells were transiently transfected with β2AR or cotransfected with β2AR plus either β-arrestin, arrestin 3, or visual arrestin and assayed for both agonist-induced internalization and down-regulation. Both β-arrestin and arrestin 3, but not visual arrestin, promoted an ~3-fold increase in agonist-induced internalization of the β2AR after 30 min of isoproterenol treatment (Fig. 1A). Previous studies have established that this effect is likely mediated via a carboxy-terminal clathrin binding domain in β-arrestin and arrestin 3 (23) that functions to recruit the receptor complex to clathrin-coated pits, resulting in receptor internalization (7).

We next determined the effect of arrestins on β2AR down-regulation in COS-1 cells. Similar to the internalization results, expression of either β-arrestin or arrestin 3, but not visual arrestin, significantly increased isoproterenol-stimulated down-regulation of the β2AR after a 24-h incubation with agonist (Fig. 1B). However, although the effect of β-arrestin and arrestin 3 appears specific, these proteins did not increase agonist-promoted down-regulation as effectively as they increase β2AR internalization. Thus, in an effort to increase the observed effect, we expressed both arrestin 3 and G protein-coupled receptor kinase 2 with the β2AR. G protein-coupled receptor kinase 2 expression augmented the effect of arrestins on agonist-stimulated internalization of the β2AR after 30 min of isoproterenol treatment (Fig. 1A). Previous studies have demonstrated that this effect is likely mediated via a carboxy-terminal clathrin binding domain in β-arrestin and arrestin 3 (23) that functions to recruit the receptor complex to clathrin-coated pits, resulting in receptor internalization (7).

We also wanted to assess whether inhibitory modulators of clathrin-mediated endocytosis would affect down-regulation of the β2AR. For these studies we chose to examine the effect of dynamin-K44A on agonist-stimulated down-regulation. Dynamin is a GTP-binding protein that plays a critical role in clathrin-coated vesicle formation (25). Previous studies have demonstrated that dynamin-K44A is deficient in GTP binding and functions as an effective dominant negative protein to potently block clathrin-mediated endocytosis (26). Indeed, dynamin-K44A overexpression in several cell lines has been demonstrated to significantly attenuate agonist-induced internalization of the β2AR (27, 28). Since COS-1 cells have a relatively low intracellular level of β2AR down-regulation, we tested the effect of dynamin-K44A on arrestin 3-promoted down-regulation in these cells. Overexpression of dynamin-K44A inhibited arrestin 3-promoted β2AR internalization ~72% (Fig. 1A), whereas an ~53% reduction in down-regulation was observed (Fig. 1B). This suggests that both β2AR internalization and down-regu-
isoproterenol-mediated internalization. Cells were harvested 48-h post-transfection and incubated with 1 μM (-)-isoproterenol at 37 °C for 30 min, washed extensively in ice-cold PBS, resuspended, and analyzed for cell surface β2AR expression as described under “Experimental Procedures.” Results represent the mean ± S.E. of 3–6 experiments performed in triplicate.

To determine whether agonist-promoted internalization via clathrin-coated pits plays a cell-specific role in β2AR down-regulation, we tested the effects of arrestins and dynamin-K44A in additional cell lines. HeLa cells appear to display many of the characteristics of agonist-induced internalization and down-regulation of the β2AR that we observed in COS-1 cells, although down-regulation was significantly higher after a 24-h treatment with isoproterenol (~48%). Coexpression of arrestin 3 with the receptor promoted agonist-mediated internalization ~2–2.5-fold, whereas coexpression of dynamin-K44A inhibited internalization ~50% (Fig. 2A). Similarly, arrestin 3 promoted and dynamin-K44A inhibited receptor down-regulation, although the effects of arrestin 3 and dynamin-K44A appeared to be more pronounced at the shorter time points (1–5 h) compared with 24 h (Fig. 2B). Notably, dynamin-K44A appeared only modestly effective at blocking agonist-induced down-regulation in these cells at 24 h. This suggests that additional pathways of intracellular protein degradation might contribute to β2AR down-regulation in HeLa cells. Such pathways could include receptor trafficking of the β2AR via caveolae, which appears to occur in A431 cells (29, 30), as well as ubiquitin-targeted protein degradation, which plays a role in rhodopsin (31) and α-mating factor receptor degradation (32).

We also assessed the effects of arrestins and dynamin-K44A on β2AR internalization and down-regulation in HEK293 cells. As previously demonstrated (6, 28), overexpression of β-arrestin only marginally promoted isoproterenol-stimulated internalization of the β2AR in HEK293 cells (Fig. 2A). Similarly, β-arrestin overexpression also had no effect on agonist-induced down-regulation of the β2AR in HEK293 cells after 24 h of isoproterenol treatment (Fig. 2B). Conversely, dynamin-K44A effectively attenuated agonist-mediated internalization of the β2AR (Fig. 3A and Refs. 27 and 28). Strikingly, inhibition of β2AR internalization by dynamin-K44A also resulted in virtually a complete blockade of β2AR down-regulation (Fig. 3B). Moreover, β-arrestin (319–418), a dominant-negative mutant that contains the carboxyl-terminal clathrin binding domain of β-arrestin (28), had an intermediate effect on both internalization and down-regulation of the β2AR (Fig. 3). The ability of the β-arrestin dominant-negative mutant to partially inhibit agonist-induced internalization and down-regulation supports the idea that arrestins are involved in β2AR internalization, and by association, in receptor down-regulation. Taken together, the results from COS-1, HeLa, and HEK293 cells indicate that a proportion of β2ARs internalized via clathrin-mediated endocytosis are targeted for down-regulation.

Presumably, the trafficking pattern for the β2AR might be similar to that seen for other G protein-coupled receptors in which activated receptors are sorted to lysosomes for degradation (11–13). Thus, we might expect that some proportion of the β2ARs that are internalized might also be sorted to lysosomes for degradation. To better visualize agonist-induced trafficking of the β2AR, we expressed a previously characterized green fluorescent protein-conjugated β2AR (β2AR-GFP) (14) in HEK293 cells. HEK293 cells transfected with β2AR-GFP were plated onto coverslips and initially incubated with rhodamine-labeled dextran, which is taken up by fluid phase endocytosis and accumulates in late endosomes and lysosomes (33). Before agonist stimulation, the fluorescence pattern of β2AR-GFP suggested localization primarily at the plasma membrane (Fig. 4A). This contrasts with the fluorescence pattern of rhodamine-labeled dextran, which has a distinct intracellular punctate appearance (Fig. 4). After a 30-min incubation with isoproterenol, β2AR-GFP was primarily visualized as a distinct punctate pattern of fluorescence (Fig. 4B). However, this staining pattern did not significantly colocalize with rhodamine-labeled dextran, suggesting that the β2AR is likely in early endocytic vesicles. Indeed previous studies have demonstrated that an epitope-tagged β2AR in HEK293 cells colocalizes with markers for early endocytic vesicles after several minutes of isoproterenol stimulation (34). In contrast, 3 h of stimulation with isoproterenol resulted in significant colocalization of β2AR-GFP and rhodamine-labeled dextran, as seen by the appearance of yellow where the green and red patterns are coincident (Fig. 4C). Colocalization of β2AR-GFP and dextran can be detected as early as 1 h after agonist addition (data not shown) and increased out to 24 h (Fig. 4D). These results are similar to
those observed in HeLa cells, where β2AR-GFP colocalizes with rhodamine-labeled transferrin, a marker for early endosomes, after short term agonist treatment, whereas colocalization of β2AR-GFP with dextran is observed with longer incubation times (>1 h) (14). Thus, these results demonstrate that in both HEK293 and HeLa cells some portion of the β2AR are sorted from early endosomes to lysosomes upon prolonged stimulation with isoproterenol, defining a mechanism for internalization-dependent, agonist-mediated degradation of the β2AR.

The mechanisms involved in β2AR down-regulation are diverse and include both translational (15–19) and transcriptional (20–22) regulation. Studies utilizing carboxyl-terminal mutants of the β2AR that disrupt its ability to internalize in an agonist-dependent manner have not demonstrated an effect on down-regulation (15, 16). Additional mutations ablate the ability of the β2AR to undergo agonist-mediated down-regulation, seemingly without affecting its ability to internalize (17–19). The assumption from these studies was that these two regulatory processes were distinct. In contrast, our data demonstrate that β2AR internalization can directly contribute to down-regulation. HEK293 cells transfected as described above were harvested at 48 h and plated onto 60-mm tissue culture dishes. Transfected cells were then treated with 10 μM (-)-isoproterenol for various times, washed extensively in PBS, lysed, and analyzed for β2AR expression as described under “Experimental Procedures.” β2AR expression levels were ~1.5–3 pmol/mg protein. Results represent the mean ± S.E. of six experiments performed in triplicate.

FIG. 3. Effect of the thrombin receptor on agonist-induced β2AR redistribution in HEK293 cells. A, effect of thrombin on β2AR redistribution in HEK293 cells. HEK293 cells transfected as described above were harvested, washed extensively in ice-cold PBS, lysed, and analyzed for cell surface β2AR expression as described under “Experimental Procedures.” Results represent the mean ± S.E. of four experiments performed in triplicate. B, effect of thrombin on β2AR redistribution in HEK293 cells. HEK293 cells transfected as described above were harvested and washed extensively in ice-cold PBS, resuspended, and analyzed for cell surface β2AR expression as described under “Experimental Procedures.” Results represent the mean ± S.E. of six experiments performed in triplicate.

FIG. 2. Effect of arrestin 3 and dynamin-K44A on agonist-induced redistribution of the β2AR in HeLa cells. A, effect of arrestin 3 and dynamin-K44A on agonist-induced redistribution of the β2AR in HeLa cells. HeLa cells were transfected with the β2AR alone or co-transfected with arrestin 3 (arr3) or dynamin-K44A (K44A) and analyzed for their ability to undergo isoproterenol-mediated internalization. Cells were harvested and treated with 1 μM (-)-isoproterenol at 37 °C for various times, washed extensively in ice-cold PBS, resuspended, and analyzed for cell surface β2AR expression as described under “Experimental Procedures.” Results represent the mean ± S.E. of six experiments performed in triplicate. β2AR expression levels were 1–2.5 pmol/mg protein. B, effect of arrestin 3 and dynamin-K44A on agonist-induced β2AR down-regulation. HeLa cells transfected as described above were harvested at 48 h and plated onto 60-mm tissue culture dishes. Transfected cells were then treated with 10 μM (-)-isoproterenol for 24 h, washed extensively in PBS, lysed, and analyzed for β2AR expression as described under “Experimental Procedures.” β2AR expression levels were ~1.5–3 pmol/mg protein. Results represent the mean ± S.E. of six experiments performed in triplicate.

A

![Graph A](image)

B

![Graph B](image)
tonic cycling or agonist-stimulated internalization of the receptor (35). It has been postulated that targeting of the thrombin receptor is dependent on the binding of an as yet undefined protein to this region of the receptor (35). For the yeast α-mating factor receptor, agonist-promoted phosphorylation and subsequent ubiquitination of the carboxyl tail appears to function as an efficient signal for internalization and trafficking of the receptor to lysosomes (32). Some growth factor receptors are also efficiently trafficked to lysosomes after activation. Perhaps the best example is the epidermal growth factor receptor, where receptor internalization and degradation is mediated by distinct endocytic and lysosomal targeting sequences (36). Recent studies have identified a protein called sorting nexin-1 (SNX1) which binds to the lysosomal targeting region of the epidermal growth factor receptor and likely plays a role in lysosomal sorting of the receptor (37). Whether lysosomal targeting of internalized β2ARs occurs randomly or via the binding of another protein remains unknown. However, it seems likely that other as yet unidentified factors will be involved in determining the fate of internalized β2ARs.

In summary, agonist-induced down-regulation of the β2AR can be mediated by the receptor to lysosomes. We have demonstrated in three cell lines that by regulating the internalization of β2AR-GFP platted onto coverslips 24 h post-transfection and incubated in the presence of 1 mg/ml rhodamine-labeled dextran overnight. Cells were stimulated with 10 μM (−)isoproterenol for 0 min (A), 30 min (B), 3 h (C), or 24 h (D). The cells shown were fixed in 3.7% formaldehyde and imaged using both fluorescein isothiocyanate and rhodamine filter sets on a confocal microscope. Images were overlaid using Photoshop software.

**FIG. 4. Visualization of isoproterenol-stimulated β2AR redistribution in HEK293 cells.** HEK293 cells expressing β2AR-GFP were plated onto coverslips 24 h post-transfection and incubated in the presence of 1 mg/ml rhodamine-labeled dextran overnight. Cells were stimulated with 10 μM (−)isoproterenol for 0 min (A), 30 min (B), 3 h (C), or 24 h (D). The cells shown were fixed in 3.7% formaldehyde and imaged using both fluorescein isothiocyanate and rhodamine filter sets on a confocal microscope. Images were overlaid using Photoshop software.

**Acknowledgments**—We thank Dr. J. Keen and members of the Benovic lab for helpful discussions, P. Hingorani for confocal microcopy, Dr. B. Kobilka for the Flag-tagged β2AR construct, and Drs. H. Damke and S. Schmid for the dynamin-K44A construct.

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J. Biol. Chem. 1998, 273:6976-6981.
doi: 10.1074/jbc.273.12.6976

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