Resistance of the Human β₁-Adrenergic Receptor to Agonist-induced Ubiquitination

A MECHANISM FOR IMPAIRED RECEPTOR DEGRADATION*

Received for publication, June 11, 2004, and in revised form, July 28, 2004
Published, JBC Papers in Press, August 25, 2004, DOI 10.1074/jbc.M406501200

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Down-regulation is a classic response of most G protein-coupled receptors to prolonged agonist stimulation. We recently showed that when expressed in baby hamster kidney cells, the human β₁ AR, but not the β₂ AR-adrenergic receptor (AR) is totally resistant to agonist-mediated down-regulation, whereas both have similar rates of basal degradation (Liang, W., Austin, S., Hoang, Q., and Fishman, P. H. (2003) J. Biol. Chem. 278, 39773-39781). To identify the underlying mechanism(s) for this resistance, we investigated the role of proteasomes, lysosomes, and ubiquitination in the degradation of β₁ AR expressed in baby hamster kidney and human embryonic kidney 293 cells. Both lysosomal and proteasomal inhibitors reduced β₁ AR degradation in agonist-stimulated cells but were less effective on basal degradation. To determine whether β₁ AR trafficked to lysosomes we used confocal fluorescence microscopy. We observed some colocalization of β₁ AR and lysosomal markers in agonist-treated cells but much less than that of β₂ AR even in cells co-transfected with arrestin-2, which increases β₁ AR internalization. Ubiquitination of β₂ AR readily occurred in agonist-stimulated cells, whereas ubiquitination of β₁ AR was not detectable even under conditions optimal for that of β₂ AR. Moreover, in cells expressing β AR chimeras in which the C termini have been switched, the chimeric β₂ AR with β₁ AR C-tail underwent ubiquitination and down-regulation, but the chimeric β₁ AR with β₂ AR C-tail did not. Our results demonstrate for the first time that β₁ AR and β₂ AR differ in the ability to be ubiquitinated. Because ubiquitin serves as a signal for sorting membrane receptors to lysosomes, the lack of agonist-mediated ubiquitination of β₁ AR may prevent its extensive trafficking to lysosomes and, thus, account for its resistance to down-regulation.

G protein-coupled receptors (GPCRs)† upon activation by agonists initiate a variety of signaling cascades that modulate cell function and metabolism (1). For most GPCRs, activation is followed by desensitization and internalization of the receptors, which then are targeted to either recycling or degradation pathways (2, 3). Several mechanisms for GPCR internalization have been described, the clathrin-coated pit endocytic pathway being the best understood. GPCR kinase-catalyzed phosphorylation of the agonist-occupied receptors promotes arrestin binding to the receptors. Arrestins also interact with clathrin and AP-2 adapter proteins to form complexes that target the receptors into the coated pits (4, 5). Based on differences in the stability and trafficking of arrestin-receptor complexes, GPCRs are separated into two classes (6, 7). Class A such as β₂ AR, α₁b AR and μ opioid receptors bind to arrestin-3 with higher affinity than arrestin-2. The interaction is transient, and upon endocytosis, the arrestin dissociates and returns to the cytosol, whereas the receptor undergoes rapid recycling. Class B such as vasopressin V2 and angiotensin AT1a receptors bind with equal affinity to arrestin-2 and -3 to form stable complexes that internalize together. The receptors recycle more slowly and are degraded more rapidly.

Recent studies indicate the involvement of ubiquitination in the internalization and degradation of GPCRs (for reviews, see Refs. 8 and 9). Conjugation of ubiquitin, a 76-amino acid polypeptide, to lysine residues of endoplasmic reticulum-retained proteins initially was found to function as a signal for their degradation by proteasomes (for a review, see Ref. 10). Subsequently, ubiquitination was shown to regulate the endocytic trafficking of several plasma membrane receptors by sorting them to lysosomes for degradation (11). Some mammalian GPCRs that are ubiquitinated include β₂ AR (12), the chemokine CXCR4 receptor (13), μ and δ opioid receptors (Ref. 14, but see Ref. 15), and the vasopressin V2 receptor (16). Eliminating receptor ubiquitination reduces agonist-mediated receptor degradation but not internalization. Thus, ubiquitination appears to function as a sorting signal for targeting the receptors for degradation in lysosomes.

Although the ubiquitination of proteins is a prerequisite for their degradation by proteasomes, the role of the latter in the degradation of GPCRs is unclear. Agonist-promoted degradation of CXCR4 receptors is blocked by inhibitors of lysosomal but not proteasomal function, and the receptors are sorted to lysosomes (13). Proteasomal inhibitors are reported to either reduce (12) or have no effect (17) on agonist-mediated internalization and degradation of β₂ AR. In one study proteasomal but not lysosomal inhibitors reduced the down-regulation of μ and δ opioid receptors (14), whereas in another, lactacystin, a very specific proteasomal inhibitor, did not impair agonist-induced internalization, lysosomal targeting, and degradation of the δ opioid receptor (15). Some of these studies found that steady-state receptor levels increase in unstimulated cells treated with...
proteasomal inhibitors (14, 17). The presence of proteasomal inhibitors also enhances the ubiquitination of some GPCRs (14, 16).

βAR, one of the three βAR subtypes, although widely distributed in various tissues, is the predominant subtype in heart and certain brain regions. βAR has a major role in regulating cardiac output in response to norepinephrine and epinephrine (18), and βAR in synaptic junctions mediates the effects of noradrenergic stimulation on long term potentiation (19). Because βAR is associated with human diseases such as congestive heart failure (18) and depression (20), the regulation of βAR is of considerable interest. In comparison to the human βAR, the human βAR is more resistant to agonist-mediated desensitization (21, 22), internalization (21, 23–27), and down-regulation (21, 23, 26, 28, 29). We have previously shown that in agonist-stimulated BHK cells, βAR is up-regulated due to its resistance to degradation and its increased synthesis (29). We recently demonstrated that βAR expressed in BHK and HEK 293 cells undergoes agonist-mediated endocytosis by the clathrin-coated pit pathway but traffics to an endosomal compartment distinct from that of βAR (27). Thus, differences in subtype degradation may be due to differences in subtype trafficking. Because ubiquitin serves as a sorting signal for lysosomal degradation of receptors, we investigated its role in the resistance of βAR to degradation. We found that in contrast to βAR, agonist-mediated ubiquitination of βAR was not detected. In addition, when we used chimeric receptors in which the C-tails of the two subtypes were exchanged, we observed ubiquitination of the β2β1c-tAR but not of the β1β2c-tAR chimeras. The inability of the C-tail of βAR to facilitate receptor ubiquitination may be the basis for its insensitivity to agonist-mediated degradation.

EXPERIMENTAL PROCEDURES

Materials—ALLN and leupeptin were from ICN, lactacystin and MG132 were from Calbiochem, chloroquine, NH2Cl, and NEM were from Sigma, and E-64 was from Roche Applied Science. LysoTracker® Red DND-99 and ProLong were from Molecular Probes. Rabbit anti-human βAR (A-20) and βAR (H-20) and mouse monoclonal anti-ubiquitin IgG1 (Ub/P4D1) were from Santa Cruz Biotechnology, Alexa Fluor-conjugated mouse anti-HA.11 was from Covance, and monoclonal anti-human LAMP1 IgG1 (H4A3) was from BD Biosciences, and Cy3-conjugated donkey anti-mouse was from Jackson ImmunoResearch. Sources of other reagents were described previously (27–29). Plasmids pCDNA3.1-h1AR, -h1γAR, -h1β1AR, -h1β2AR, -h1β3AR, -h1γ1AR, pE-h1AR, -h1ct-AR, pE-h1AR, pCDNA3-h1AR, GFP, -arrestin-2 and -3 were described before (27, 29).

Cell Culture and Transfection—BHK (clone tk−) and HEK 293 cells were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Clonal lines stably expressing either βAR or βAR or co-expressing both arrestin-2 and either subtype were described previously (27, 29). To obtain cells expressing wild type or chimeric HA-βAR or βAR-GFP or co-expressing both arrestin-2 and either subtype, cells were transfected with pcDNA3.1-h1AR, -h1γAR, -h1β1AR, -h1β2AR, -h1β3AR, -h1γ1AR, pE-h1AR, -h1ct-AR, and -h1β2AR, pE-h1AR, pCDNA3-h1AR, GFP, -arrestin-2 and -3 were described before (27, 29).

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RESULTS

βAR Resistance to Agonist-mediated Down-regulation in HEK 293 Cells—We recently showed that human βAR expressed in BHK cells is up-regulated by persistent agonist stimulation due to a cAMP-mediated increase in βAR mRNA levels and the resistance of βAR to agonist-mediated degradation (29). To further investigate the regulation of human βAR, we used the HEK 293 cell line, which is well characterized and serves as a model for studying the function and regulation of various human proteins. HEK 293 cells stably expressing either βAR or βAR were exposed to 10 μM ISO at 37 °C for up to 24 h and then assayed for total βAR binding activity using [125I]iodocyanopindolol. The number of βAR binding sites remained unchanged, whereas that of βAR decreased to 64% that of control with a t½ of 6.8 h (Fig. 1A). We have shown that overexpressing arrestin-2 and -3 increases agonist-mediated internalization of βAR in BHK and HEK 293 cells (27). Enhancement of βAR internalization by arrestin-2, however, does not lead to down-regulation of βAR in BHK cells (29). Several studies indicated that arrestin-3 has a larger role than...
arrestin-2 in the regulation of β2AR (7, 12). Therefore, we investigated the effects of arrestin-3 on prolonged agonist-mediated regulation of β1AR in BHK and HEK 293 cells. As shown in Fig. 1B, total β1AR binding activity was not reduced by persistent agonist treatment regardless of arrestin-3 co-expression. Thus, although both arrestins facilitate the internalization of β1AR, neither has an effect on its degradation.

Degradation or turnover of the receptor proteins in HEK 293 cells was determined by measuring the disappearance of biotinylated cell surface βAR using immunoprecipitation and streptavidin overlay as described under “Experimental Procedures.” In the absence of agonist, the basal degradation of both β-subtypes was similar, with ~40% of the biotin-labeled receptors being lost in 4 h (Fig. 2). In cells treated with agonist for 4 h, degradation of β2AR increased more than that of β1AR. Based on these results, β2AR expressed in HEK 293 cells is resistant to agonist-mediated down-regulation as was observed in BHK cells (27). Thus, the regulation of human β1AR is similar in both cell lines and is significantly different from that of human β2AR.

Effects of Lysosomal and Proteasomal Inhibitors on Degradation of β1AR—To determine whether proteasomes and lysosomes, two major sites for protein degradation, are involved in β1AR degradation, we used a series of known inhibitors. Chloroquine and NH4Cl inhibit protease activity in lysosomes by neutralizing the acidic pH (31). E-64 and leupeptin inhibit lysosomal cysteine proteases, and leupeptin also inhibits serum proteases (32). ALLN, MG132, and lactacystin block proteasomal activity, with lactacystin having the greatest specificity (17). Degradation of β1AR expressed in BHK and HEK 293 cells was determined using the surface biotinylation procedure described above. The biotinylated cells were pretreated with the inhibitors and then incubated in the presence or absence of ISO for 4 h. As shown in Table I, agonist treatment only had a significant effect on HEK 293 cells not exposed to inhibitor. All the inhibitors reduced the basal β1AR degradation in BHK cells, but only chloroquine, E-64, and ALLN reached statistical significance, with the effect of ALLN being substantial. ALLN, followed by MG132 and chloroquine, significantly lowered the agonist-mediated degradation. The degradation of β1AR in agonist-stimulated HEK 293 cells was significantly inhibited by NH4Cl, E-64, and lactacystin, but only the latter had an effect on basal degradation. These results suggest that β1AR degradation is more sensitive to proteasomal and lysosomal inhibitors in agonist-stimulated cells. They further suggest that basal receptor turnover may involve some other mechanism such as plasma membrane degradation (17). None of the inhibitors totally blocked β1AR degradation at the concentrations used. It should be noted, however, that higher concentrations often led to cell death (especially HEK cells).

Differences in Extent of β1AR and β2AR Trafficking to Lysosomes in Agonist-stimulated Cells—To further establish that β1AR undergoes some lysosomal degradation, we visualized the intracellular trafficking of β1AR as well as β2AR using confocal fluorescence microscopy. HEK 293 cells stably expressing HA-tagged β2AR were stained at 4 °C with fluorescent-tagged anti-HA antibody, then washed, warmed up to 37 °C in the absence and presence of agonist for 2 h, and stained with LysoTracker® Red at the end of the 2-h period. In the unstimulated cells, HA-β2AR (green) was confined to the cell surface, and LysoTracker® Red (red) appeared in cytoplasmic vesicles that are presumably lysosomes (Fig. 3A). Because β1AR is resistant to agonist-mediated internalization, most of the receptors remained at the plasma membrane of stimulated cells, and only a small fraction was translocated into the cytosol (Fig. 3B). As
Effects of proteasomal and lysosomal inhibitors on the basal and agonist-mediated degradation of β²AR in BHK and HEK 293 cells

Cells were biotinylated at 4 °C and incubated at 37 °C in medium containing the indicated inhibitor for 30 min and then for 4 h in the presence or absence of 10 μM ISO. Cells were lysed and analyzed for biotinylated β²ARs as described in the legend to Fig. 2. Values are expressed as percent degraded and are the means ± S.E. of 3–8 independent experiments. Cells not incubated after biotinylation were used to determine 0% degradation.

| Inhibitor | BHK cells | HEK 293 cells |
|-----------|-----------|---------------|
|           | − ISO     | + ISO         | − ISO         | + ISO         |
| None      | 10 ± 2.9% | 11 ± 2.0%     | 43.7 ± 2.6%   | 39.8 ± 1.8%   |
| 10 mM NH₄Cl | 26.8 ± 7.1 | 35.4 ± 2.3%   | 36.5 ± 2.8%   | 41.4 ± 4.2%   |
| 0.1 mM chloroquine | 18.0 ± 2.8% | 38.1 ± 6.0%   | ND           | ND           |
| 0.1 mM E-64 | 18.0 ± 2.8% | 38.1 ± 6.0%   | ND           | ND           |
| 0.1 mM leupeptin | 33.4 ± 3.0 | 43.5 ± 1.4%   | 36.1 ± 0.9%   | 36.1 ± 0.9%   |
| 0.1 mM ALLN | 9.8 ± 7.4% | 8.4 ± 7.4%    | ND           | ND           |
| 0.05 mM MG132 | 19.6 ± 3.4% | 19.6 ± 3.4%   | ND           | ND           |
| 0.01 mM lactacystin | 32.1 ± 3.2 | 32.1 ± 3.2%   | ND           | ND           |
|           | 30.2 ± 4.2% | 31.2 ± 5.1%   | ND           | ND           |

* p < 0.05; two-way analysis of variance with Bonferroni’s post-test.
* Compared to cells not treated with ISO. Differences ± ISO for all other treatments are not significant.
* Compared to cells not treated with an inhibitor (none).
* ND, not determined.
* p < 0.001; two-way analysis of variance with Bonferroni’s post-test.

Effect of Arrestin on β²AR Trafficking to Lysosomes—Although overexpressing arrestin increases β²AR internalization without affecting β²AR degradation, it may influence receptor trafficking to lysosomes. In this regard, the limited endocytosis of β²AR observed in Figs. 3 and 4 may mask the extent of its trafficking to lysosomes. To explore this possibility, we examined the effects of prolonged agonist treatment on the distribution of β²AR-GFP co-expressed with arrestin-2 in HEK 293 cells and, for comparison, β²AR-GFP expressed in BHK cells. In both HEK 293 and BHK cells exposed to agonist for 24 h, we observed a substantial redistribution of β²AR-GFP from the plasma membrane to cytoplasmic vesicles, a number of which contained the lysosomal marker (Fig. 5, A–D). The β²AR-GFP-containing lysosomes, however, formed smaller, more diffuse aggregates compared with the larger, more compact aggregates containing β²AR-GFP in BHK (Fig. 5F) or HEK 293 (Fig. 4F) cells.

Agonist-stimulated Ubiquitination of β²AR but Not β²AR—Because ubiquitination was found to be involved in regulating...
the endocytosis and sorting of some GPCRs including β2AR to lysosomes (12, 13, 16), we investigated whether β1AR also is ubiquitinated. We stripped and re-probed blots similar to the one shown in Fig. 2A using a monoclonal anti-ubiquitin antibody. We were unable to detect any ubiquitinated β2AR, whereas we found ubiquitinated β1AR (data not shown). Because several studies found that ubiquitination of receptors is more readily detected in cells treated with proteasomal inhibitors, we repeated the biotinylation experiments using cells treated with and without of lactacystin, prepared duplicate blots of the immunoprecipitated biotinylated βARs, and probed one with anti-ubiquitin and the other with streptavidin. Ubiquitinated β2AR was detected in HEK 293 cells, and its level was substantially enhanced by lactacystin treatment and further increased by ISO stimulation (Fig. 6). Ubiquitinated β1AR was not detected in HEK 293 or BHK cells even in the presence of lactacystin and agonist (top panel). β1AR was present in these samples, some of which appeared as dimers (bottom panel). Shenoy et al. (12) found that β2AR ubiquitination is transient, increasing upon agonist stimulation, and then decreasing, and the presence of NEM, an inhibitor of deubiquitinating enzymes, during cell lysis and immunoprecipitation enhanced the detection of ubiquitinated proteins. We exposed lactacystin-treated HEK 293 cells to ISO for increasing times over a 4-h period and added 10 mM NEM to the lysis and RIPA buffers. Even under these optimal conditions, ubiquitination of β2AR still was not detected, whereas ubiquitination of β1AR was observed in unstimulated cells and increased upon agonist stimulation (Fig. 7). Taken together, these results clearly demonstrate that ubiquitination of β1AR does not occur in comparison with the extensive ubiquitination of β2AR in the same cells.

Carboxyl Tails of βARs Dictate Subtype-specific Ubiquitination—We have shown that the C termini of β1AR and β2AR determine subtype specificity in agonist-mediated phosphorylation, internalization, and prolonged regulation in BHK cells (29). We expressed the same chimeras in which the C-tails of β1AR and β2AR were exchanged (β2/β1ct-AR and β1/β2ct-AR) as well as the wt-βARs in HEK 293 cells and determined the extent of agonist-mediated internalization of surface receptors and regulation of total receptors (Fig. 8A and B). The results were similar to those obtained in BHK cells. The β2/β1ct-AR chimera exhibited increased internalization and down-regulation compared with wt-β2AR, whereas the responses of the β2/β1ct-AR chimera were reduced compared with wt-β2AR. We then explored the possibility of a role of the C-tails in β-subtype ubiquitination. The cells were treated with ISO for up to 24 h and analyzed for βAR ubiquitination (Fig. 8C). ISO stimulation resulted in a time-dependent increase in the ubiquitination of wt-β2AR and chimeric β2/β1ct-AR, which lasted up to 24 h. In contrast, no ubiquitinated wt-β1AR or chimeric β2/β1ct-AR was detected. These results indicate that the C-tails play a major role in controlling the ubiquitination and degradation of the two subtypes and provide additional evidence that the two processes are closely linked.

Co-detection of β2AR but Not β1AR Proteins by Anti-ubiquitin and β2AR Antibodies—Although anti-β2AR antibodies were used for the immunoprecipitations and the streptavidin overlay confirmed that β2ARs from biotinylated cells were in the

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**Fig. 5.** Effect of arrestins on agonist-mediated βAR internalization and trafficking to lysosomes. HEK 293 (A and B) and BHK (C–F) cells stably co-expressing arrestin-2 and β2AR-GFP (A–D) or β1AR-GFP (E and F) were incubated at 37 °C in the presence (B, D, and F) or absence (A, C, and E) of 10 μM ISO for 24 h. LysoTracker® Red was added at 23.5 h. The cells were washed, fixed, and visualized with a confocal microscope for the distribution of receptors (green) and LysoTracker® Red (red). Colocalization is shown in yellow. Bar, 5 μm.

**Fig. 6.** Effects of lactacystin and agonist on ubiquitination of βARs. Cells stably expressing β1AR or β2AR were biotinylated, incubated in the presence and absence of 10 μM ISO and 10 μM lactacystin as indicated, then were lysed and extracted in RIPA buffer. Soluble βARs were immunoprecipitated with anti-βAR antibodies (IP/βAR) and subjected to SDS-PAGE and immunoblotting with anti-ubiquitin (IB:Ub) (upper panel) or blotting with peroxidase-conjugated streptavidin (B:StAv) (lower panel) as described under “Experimental Procedures.” Shown are representative blots from one of three independent experiments.

**Fig. 7.** Time course for agonist-stimulated ubiquitination of βARs in HEK 293 cells. Cells expressing β1AR or β2AR were exposed for 30 min to 10 μM lactacystin and for the indicated times to 10 μM ISO and analyzed for ubiquitination of βARs as described in the legend to Fig. 6 except 10 mM NEM was added to the lysis buffer to inhibit any deubiquitinating enzymes. Shown is a representative blot (WB) from one of three independent experiments. IP, immunoprecipitation.
precipitats (Fig. 6), it was possible that the ubiquitin-conjugated proteins detected were not \( \beta_2 \)AR but proteins that co-precipitated with \( \beta_2 \)AR. To address this possibility, we probed blots similar to the one probed with the anti-ubiquitin antibody (Fig. 8C) with anti-\( \beta_1 \)AR and -\( \beta_2 \)AR antibodies instead (Fig. 8D). The anti-\( \beta_2 \)AR antibodies detected immune-reactive proteins between 75 and 200 kDa that corresponded to the proteins revealed by the anti-ubiquitin antibody. Co-detected proteins were only observed in immunoprecipitates from cells expressing wt-\( \beta_2 \)AR or the \( \beta_1/\beta_2 \)ct-AR chimera. Even though the anti-\( \beta_2 \)AR antibodies reacted with forms of \( \beta_2 \)AR with high apparent molecular masses, no corresponding proteins were detected in blots probed with the anti-ubiquitin antibody. These observations further confirm that \( \beta_2 \)AR but not \( \beta_1 \)AR undergoes ubiquitination.

**DISCUSSION**

In the present study we found that the human \( \beta_1 \)AR stably expressed in HEK 293 cells was resistant to agonist-mediated down-regulation, in agreement with our earlier work using BHK cells (29). In contrast, the human \( \beta_2 \)AR undergoes agonist-mediated down-regulation in both cell lines. We identified another major difference between the two subtypes; \( \beta_2 \)AR but not \( \beta_1 \)AR underwent ubiquitination. Ubiquitination of \( \beta_1 \)AR was observed in unstimulated cells, was increased in a time-dependent manner by agonist stimulation, and was enhanced by lactacystin treatment. We were unable to detect ubiquitination of \( \beta_1 \)AR even under optimal conditions for that of \( \beta_2 \)AR. In addition, we found that the C-tails of \( \beta_1 \)AR and \( \beta_2 \)AR were key determinants of ubiquitination, as the chimeras, \( \beta_1/\beta_2 \)ct-AR and \( \beta_1/\beta_2 \)ct-AR, in which the C-tails are switched, did or did not undergo ubiquitination, respectively. The C-tails are also major determinants of agonist-mediated phosphorylation, internalization, degradation, and down-regulation of the two subtypes (Ref. 29 and the present study). \( \beta_1/\beta_2 \)ct-AR undergoes these processes to a greater extent than does \( \beta_1/\beta_2 \)ct-AR, the latter being almost as resistant to down-regulation as is wt-\( \beta_1 \)AR. Taken together, these results indicated a strong relationship between ubiquitination and down-regulation. They also are consistent with other studies of GPCRs including \( \beta_2 \)AR (12, 13, 16).

Receptor ubiquitination requires receptor phosphorylation (12, 13, 34). For \( \beta_1 \)AR, ubiquitination depends not only on GPCR kinase-catalyzed phosphorylation of the receptor C-tail but also on binding of arrestin, which recruits ubiquitinating enzymes to the complex (12). In cells expressing a phosphorylation-defective \( \beta_1 \)AR or lacking arrestins, ubiquitination of \( \beta_1 \)AR is not observed. We propose that the role of phosphorylation in the ubiquitination of \( \beta_1 \)AR is only to facilitate arrestin binding. This proposal is strongly supported by our current and previous findings as well as those of others. First, agonist stimulation increases the phosphorylation of \( \beta_1 \)AR only slightly compared with that of \( \beta_2 \)AR (27, 29, 33). Second, arrestins interact more transiently with \( \beta_1 \)AR than \( \beta_2 \)AR in agonist-stimulated cells (25). Third, the stability of the interaction of arrestins with \( \beta_1 \)AR is increased when the C-tail is from \( \beta_2 \)AR (25), and agonist-stimulated phosphorylation (29) and ubiquitination (present study) of the two subtypes was determined by the C-tail. As a consequence of the limited agonist-stimulated phosphorylation of \( \beta_1 \)AR, the interaction of arrestin with the receptor may be too weak for arrestin to recruit ubiquitinating enzymes, and thus, \( \beta_1 \)AR is not ubiquitinated. We believe that there is a clear sequential relationship between agonist-stimulated phosphorylation of the receptor C-tail and high affinity arrestin binding, ubiquitination, and trafficking to lysosomes to be degraded.

We recently established that although human \( \beta_1 \)AR and \( \beta_2 \)AR undergo agonist-stimulated endocytosis through clathrin-coated pits, each subtype traffics to a different endosomal compartment (27). Our current results may explain this divergence. Conjugation of membrane receptors with ubiquitin is a signal for sorting of the receptors to lysosomes after endocytosis (11, 12, 35, 36). Sorting involves Hrs, a mammalian ortholog of a yeast vacuolar sorting protein, which has both clathrin and ubiquitin binding domains and associates with clathrin-coated microdomains on endosomes (36). These endosomes are morphologically and functionally distinct from clathrin-positive and Hrs-negative early endosomes. Transferrin receptors are not ubiquitinated and, after endocytosis, appear in the Hrs-negative endosomes and rapidly recycle to the plasma membrane. Transferrin receptor-ubiquitin fusion proteins localize to Hrs-positive endosomes and are sorted to the degradative pathway. The chemokine receptor CXCR4, which undergoes agonist-promoted ubiquitination, was shown to colocalize with Hrs-positive endosomes (37). Therefore, we propose that the non-ubiquitinated \( \beta_1 \)AR traffics to Hrs-negative endosomes and is preferentially sorted to the recycling pathway, whereas the ubiquitinated \( \beta_2 \)AR localizes to Hrs-positive endosomes and is targeted to lysosomes for degradation.

Although ubiquitin functions as a signal for sorting a number of GPCRs to lysosomes, other sorting mechanisms have been described (38). In agonist-stimulated cells, the \( \delta \) opioid receptor is rapidly degraded, whereas the \( \mu \) opioid receptor is recycled, and exchanging their C-tails generates chimeras that, respectively, are recycled and degraded. GASP, a protein that binds to the C-tail of the \( \delta \) opioid receptor, has been identified as lysosomal-sorting protein (39). A mutated \( \delta \) opioid receptor, in which all the cytoplasmic lysine residues were replaced with arginines is internalized, sorted to lysosomes and degraded.
similar to the wild type receptor (15). In addition, a recycling sequence has been identified in the C-tail of the μ receptor, which when deleted results in a mutated receptor that is rapidly degraded and, when fused to the δ receptor, confers recycling activity on that subtype (40). Thus, we cannot rule out the possibility that βAR may have an analogous sequence in its C-tail that targets the receptor to the recycling pathway as opposed to recycling by default, i.e. in the absence of a positive lysosomal-sorting signal such as ubiquitination or interaction with a GASP-like protein.

Receptors are constantly being turned over and replaced by newly synthesized receptors at rates that maintain the steady state (41). In the absence of agonist, both subtypes turned over at similar rates (t1/2 of ∼6.5 h in BHK and ∼5 h in HEK 293 cells). In the presence of agonist, the degradation rate of βAR was increased negligibly in BHK and modestly in HEK 293 cells, whereas that of β2AR was more than doubled in both cell lines. Both lysosomal and proteasomal inhibitors reduced βAR degradation in agonist-treated BHK and HEK 293 cells. Because β2AR is not ubiquitinated, the effects of the proteasomal inhibitors remain to be clarified. The basal degradation of βAR was substantially inhibited only by ALLN, whereas lactacystin, a more specific inhibitor of proteasomal function, was less effective in both cell lines. More likely, basal turnover of βAR occurs elsewhere, such as at the plasma membrane (17). Consistent with this possibility, we observed that fluorescent-labeled anti-HA antibody bound to cell surface HA-β2AR expressed in HEK 293 cells did not accumulate inside the unstimulated cells after 2 h at 37 °C (Fig. 3A). In addition, there was very little accumulation of fluorescence inside unstimulated HEK 293 and BHK cells expressing β1AR-GFP (Fig. 4A; Fig. 5, A and C). Based on a different approach, a similar proposal was made that basal and agonist-stimulated degradation of the vasopressin V2 receptor involves two separate processes (16).

To detect any agonist-promoted trafficking of βAR to lysosomes, we used confocal fluorescence microscopy to visualize both receptors and lysosomes. Colocalization of very small amounts of βAR and two different lysosomal markers was observed in agonist-stimulated HEK 293 cells by 2–4 h. By comparison, agonist-promoted trafficking of β2AR to lysosomes in HEK 293 cells was observed by 1 h and was much more robust. In addition, lysosomes containing β2AR appeared as large aggregates, whereas βAR-associated lysosomes appeared less aggregated, smaller, and more diffuse. In these experiments, β2AR mostly redistributed from the plasma membrane to the cytosol, whereas the distribution of βAR was the opposite. In cells overexpressing arrestin-2, the translocation of β1AR from membrane to cytosol increased, as did its accumulation in lysosomes. Accumulation of β2AR in lysosomes, however, was more extensive even though internalization of the two subtypes was similar. Despite the increase in internalization and lysosomal trafficking, β1AR remained resistant to down-regulation.

We previously showed that overexpressing arrestin-2 does not alter degradation of βAR or β2AR in agonist-stimulated BHK cells (29). Together with our present results, it appears that increasing βAR internalization has little effect on βAR degradation. This raises an interesting point, as upon agonist stimulation, a fraction of each subtype is translocated from the site of basal turnover to clathrin-coated pits and subsequent endocytosis. Degradation continues, however, at a similar rate for β2AR and at twice the rate for βAR. The reduction in β2AR degradation by lysosomal inhibitors, the trafficking of β2AR to lysosomes, and the degradation of β2AR after clathrin-mediated endocytosis all appear to be contradictory to our finding that β1AR is not ubiquitinated. A comparison of the rates of internalization and degradation provides some perspective (Table II). In both cell lines, the limited agonist-promoted internalization of βAR means that most of the receptors continue to undergo basal degradation. Therefore, the rate of βAR degradation through an agonist-mediated pathway is quite low compared with the rate of internalization. For every 1000 receptors internalized, only 24–40 are degraded, and the rest are recycled. In BHK cells overexpressing arrestin-2, the large increase in βAR endocytosis reduces the number to 6 per 1000. The agonist-mediated component of degradation, however, is almost twice that in BHK cells not overexpressing arrestin-2. Although the ratio is the same for both subtypes expressed in HEK 293 cells, the rates of agonist-mediated internalization and degradation of β2AR are twice those of βAR. These results are consistent with the observed range of trafficking of the two subtypes to lysosomes. They also suggest that the sorting machinery is not completely efficient. Thus, small amounts of non-ubiquitinated βAR may traffic to lysosomes to be degraded, whereas most will be targeted to the recycling pathway.

In summary, we have identified a major difference in the regulation of human β1AR and β2AR, namely that the latter is ubiquitinated, and the former is not. This provides a mechanism for understanding the resistance of β2AR to degradation and down-regulation and the divergent endosomal trafficking of the two subtypes after clathrin-mediated endocytosis.

Acknowledgments—We thank David Recinos and Jeremy Johnson for technical assistance, Sharon Wu for assistance in preparing the manuscript, and Dr. Carolyn Smith, manager of the NINDS Light Imaging Facility, for assistance with the confocal microscopy.

Data derived from internalization and degradation assays were fitted to one-phase exponential decay curves as described under "Experimental Procedures." Initial rates (%/min) were extrapolated as previously described (28). Values for βAR internalized (% max) have been reported previously (29). The following assumptions were made to calculate the basal and agonist components of the degradation rate in agonist-treated cells; receptors remaining on the cell surface are degraded at the basal rate, and the sum of the basal and agonist components equals the degradation rate. All values are the mean ± S.E. of three to six separate experiments.

### Table II

| Parameter                  | BHK 293  | Arr2-BHK    | HEK 293  |
|---------------------------|----------|-------------|----------|
| βAR internalized (% max)  | +        | 15.6 ± 1.3  | 13.7 ± 1.6 | 25.8 ± 1.8  |
| Rate internalized (%/min) | +        | 3.40 ± 0.62 | 9.15 ± 0.96 | 22.6 ± 1.42  |
| Basal rate degraded (%/min)| −        | 0.177 ± 0.02 | 0.168 ± 0.04 | 0.231 ± 0.02  |
| Agonist rate degraded (%/min)| +        | 0.231 ± 0.02 | 0.389 ± 0.05 | 0.231* ± 0.02  |
| Basal component           |          | 0.149 ± 0.08 | 0.104    | 0.065 ± 0.060 |
| Agonist component         |          | 0.406 ± 0.38 | 0.285    | 0.661 ± 0.329 |
| Internalized/degraded     |          | 41.5 ± 12   | 32.1 ± 4  | 155 ± 23   |

* As overexpressing arrestin-2 has only modest effects on βAR degradation, the same rates obtained with BHK cells were used.

* Ratio of rate internalized to agonist component of rate degraded.
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J. Biol. Chem. 2004, 279:46882-46889. doi: 10.1074/jbc.M406501200 originally published online August 25, 2004

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