Prolonged agonist stimulation results in down-regulation of most G protein-coupled receptors. When we exposed baby hamster kidney cells stably expressing the human $\beta_1$-adrenergic receptor ($\beta_1$AR) to agonist over a 24-h period, we instead observed an increase of $\sim$30% in both $\beta_1$AR binding activity and immune-detected receptors. In contrast, $\beta_2$AR expressed in these cells exhibited a decrease of $\sim$50%. We determined that the basal turn-over rates of the two subtypes were similar ($t_1/2 \sim 7$ h) and that agonist stimulation increased $\beta_1$AR but not $\beta_2$AR turnover. Blocking receptor trafficking to lysosomes with bafilomycin A$_2$ had no effect on basal turn-over of either subtype but blocked agonist-stimulated $\beta_1$AR turnover. As $\beta_1$AR mRNA levels increased in agonist-stimulated cells, $\beta_1$AR up-regulation appeared to result from increased synthesis with no change in degradation. To explore the basis for the subtype differences, we expressed chimeras in which the C termini had been exchanged. Each chimera responded to persistent agonist stimulation based on the source of its C-tail; $\beta_1$AR with a $\beta_2$AR C-tail underwent down-regulation, and $\beta_2$AR with a $\beta_1$AR C-tail underwent up-regulation. The C-tails had a corresponding effect on agonist-stimulated receptor phosphorylation and internalization with the order being $\beta_2$AR > $\beta_1$AR with $\beta_2$AR C-tail > $\beta_2$AR with a $\beta_1$AR C-tail > $\beta_1$AR. As internalization may be a prerequisite for down-regulation, we addressed this possibility by co-expressing each subtype with arrestin-2. Although $\beta_1$AR internalization was increased to that of $\beta_2$AR, down-regulation still did not occur. Instead, $\beta_1$AR accumulated inside the cells. We conclude that in unstimulated cells, both subtypes appear to be turned over by the same mechanism. Upon agonist stimulation, both subtypes are internalized, and $\beta_2$AR but not $\beta_1$AR undergoes lysosomal degradation, the fate of each subtype being regulated by determinants in its C-tail.

The three $\beta$-adrenergic receptor ($\beta$AR) subtypes, $\beta_1$AR, $\beta_2$AR, and $\beta_3$AR, are members of the G protein-coupled receptor (GPCR) superfamily. Although all three subtypes respond to norepinephrine and epinephrine by activating adenyllyl cyclase, they differ in their distribution, regulation, and interaction with other signaling pathways. This is evident in the heart where $\beta_1$AR is the predominant subtype followed by $\beta_2$AR and $\beta_3$AR, and each subtype appears to differ in signaling properties (1–4). $\beta_2$AR and $\beta_3$AR couple to $\mathrm{G}_i$ and $\mathrm{G}_\alpha$, whereas $\beta_3$AR only couples to $\mathrm{G}_i$. Overstimulation of $\beta_3$AR is pro-apoptotic whereas $\beta_2$AR stimulation is anti-apoptotic. During chronic heart failure, the compensating increase in sympathetic drive leads to high catecholamine levels and chronic $\beta$-adrenergic signaling, in particular the norepinephrine/$\beta_1$AR component contributes to the progression of the disease (5). Because $\beta_1$AR is implicated in chronic heart failure, there is considerable interest in its regulation.

Persistent agonist stimulation also leads to receptor down-regulation. Down-regulation is defined as a reduction in the total number of receptors and is usually determined by loss of binding sites and often attributed to receptor proteolysis. Although the down-regulation of $\beta_2$AR has been studied extensively, the mechanisms and pathways remain unresolved, which has led to the proposal of several models (6). A major question is whether receptor down-regulation occurs in the absence of internalization or is dependent on endocytosis and trafficking to lysosomes. Some studies show that internalization of $\beta_2$AR is necessary for its degradation. Following agonist-mediated endocytosis via clathrin-coated pits, most of the receptors are recycled, but some enter the lysosomal pathway and are degraded (7, 8). Other studies, however, indicate that internalization and down-regulation of $\beta_3$AR are independent of each other. Certain mutations of $\beta_3$AR impair one process but not the other (9–12), and inhibition of endocytosis does not block the degradation of $\beta_2$AR in mouse L cells or A431 cells (13). Based on a kinetic analysis, a two-pathway model of $\beta_2$AR down-regulation has been proposed: a high-affinity, low-capacity, internalization-independent pathway and a low-affinity, high-capacity, internalization-dependent pathway (14). An additional mechanism observed in some cells is the cyclic AMP-mediated reduction in steady-state $\beta_3$AR mRNA levels (15–18). The decrease is because of destabilization of the transcripts by proteins that bind to specific sequences in the 3′-untranslated region. Although some of these differences may be cell-specific, the precise mechanism of $\beta_3$AR down-regulation is not fully understood.

Less is known about the down-regulation of $\beta_1$AR, but in general it is not as responsive to this type of regulation compared with $\beta_2$AR. In rat C6 glioma cells exposed to the agonist horseradish peroxidase; $\mathrm{ICYP}$, $\mathrm{[125I]}$iodocyanopindolol; ISO, isoproterenol; HEK, human embryonic kidney; HA, hemagglutinin.
ISO, down-regulation of both subtypes occurs at similar rates (19, 20), but when the cells are exposed to atypical agonists, only β2AR levels are reduced (19). When rat H9c2 heart cells are treated with agonist, β2AR but not β1AR undergoes down-regulation (21). Distinctions also are found in vivo. Down-regulation is greater for β2AR than β1AR in fat cells isolated from dogs with chronically elevated plasma catecholamines (22) and in myocardial tissue from rats infused with norepinephrine (23). The opposite, however, was found in tissue from human failing hearts (24). The two subtypes have been directly compared by heterologously expressing each in the same cell line. β1AR is more resistant to agonist-mediated down-regulation than β2AR in CHW (25–27) and HEK 293 cells (28) and in some studies, undergoes up-regulation in the latter cells (29, 30).

We initiated the present studies to identify the mechanisms involved in the resistance to down-regulation of human β1AR during persistent agonist stimulation. As β2AR also is more resistant than β1AR to agonist-mediated internalization (25, 26, 28, 31, 32), we determined whether the latter contributed to its resistance to down-regulation. In addition, we investigated whether the two subtypes differed in basal or agonist-mediated turnover and whether basal and agonist-mediated turnover involved different pathways. Finally, as the C-tails of GPCRs including the β2AR have been identified as important determinates of sorting between recycling and degradation (33–36), we examined the effects of exchanging the C-tails of the two subtypes on receptor regulation. Our results indicate that in BHK cells, β1AR is resistant to agonist-mediated down-regulation and instead undergoes up-regulation as does its mRNA; increased β2AR internalization does not result in down-regulation; basal turnover is similar for both subtypes and appears to be non-lysosomal whereas agonist-mediated turnover of β2AR is lysosomal; and finally the C-tails are key determinants of down-regulation, the β1AR C-tail conferring resistance and the β2AR conferring C-tail susceptibility.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methotrexate was purchased from Sigma. Baflofinycin A1 was from Calbiochem. LipofectAmine Plus, G418, and TRIzol reagent were from Invitrogen. [32P]Orthophosphate and [α-32P]dCTP (3000 Ci/mmol) were from ICN. Rabbit anti-β2AR IgG (H-20) was from Santa Cruz Biotechnology. Mouse monoclonal anti-arrestin antibody (F4C1) H9252 was obtained from J. Benovic (Thomas Jefferson University, Philadelphia, PA). We generated Zem229-Arr2 by excising the coding region of the bovine arrestin-2 cDNA with EcoRI and inserting it into the corresponding sites created in the BamHI cloning site of Zem229, which has a dihydrofolate reductase selectable marker. The HA-tagged wild-type h1AR and -h2AR cDNA constructs were obtained from Thomas Jefferson University, Philadelphia, PA. We generated Zem229-Arr2 by excising the coding region of the bovine arrestin-2 cDNA with NotI and Apol and inserting it into the corresponding sites created in the BamHI cloning site of Zem229, which has a dihydrofolate reductase selectable marker.

**Cell Culture and Transfection**—The BHK cell line (clone tk-c13) was 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 β1AR or β2AR or co-expressing both arrestin-2 and either subtype were obtained by transfecting the cells with one of the Zem228c-hβ2AR plasmids or co-transfecting with both Zem228c-hβ1AR and Zem229-Arr2, selecting for resistance to G418 or both G418 and methotrexate, and by limiting dilution of the resistant cultures (26). Arrestin-2 expression was confirmed by Western blotting. To obtain cells expressing wild-type or chimeric HA-βARs, cells were transfected with one of the pcDNA plasmids using LipofectAMINE Plus according to the manufacturer’s instructions and either used after 24 h or selected with G418. Although the resistant cultures were not cloned, even expression levels remained fairly constant throughout the experimental period. All stably transfected cells were maintained in the culture medium containing 0.2 mg/ml G418 and/or 2 μM methotrexate.

**βAR Binding Assays**—Control and treated cells were assayed for total and surface receptors as described previously (27, 37). Briefly, cells grown in 6-well plates or 35-mm dishes were incubated with 10 μM ISO at 37 °C for increasing times up to 24 h, rinsed with ice-cold Ca2+ - and Mg2+-free DPBS, and lysed in 1 ml Tris-HCl, 2 mM EDTA, 1 mM EGTA, pH 7.4, and protease inhibitors at 4 °C. Portions of the lysates were assayed for protein and for total βAR activity with 250 μM [3H]ICYP for 1 h at 37 °C. Portions also were dissolved in SDS sample buffer and used for Western blotting as described below. To measure surface βAR, cells grown in 24-well plates were exposed at 37 °C for increasing times up to 30 min or to 10 μM ISO for up to 24 h. The plates were placed on a bed of ice, rapidly washed twice with ice-cold DPBS, and exposed to 5 μl [3H]ICGP-11177 for 4 °C for 1 h. Finally, the cells were washed as above and assayed for [3H] and protein. For both assays, nonspecific binding was determined in the presence of 10 μM propranolol.

**βAR mRNA Assay**—Control and agonist-treated cells were washed twice, scraped into ice-cold DPBS, and centrifuged. The cell pellet was suspended in TRIzol reagent (5–10 × 10^6 cells/ml), mixed well with 0.1% volume of chloroform, and centrifuged for 15 min at 12,000 × g at 4 °C. The clear upper layer containing RNA was removed, adjusted to 35% ethanol, and further purified using an RNeasy kit (Qiagen). First strand cDNA in 20 μl was generated from 500 ng of total RNA with Random Decamers using a RETROscript kit and protocol (Ambion). Portions (0.2–0.4 μl) of the cDNA were used to quantify the relative amounts of βAR mRNA by generating [α-32P]-labeled PCR products using a QuantumRNA 18 S Internal Standards kit and protocol (Ambion). For β1AR and β2AR, β-actin mRNA was assayed for control and treated cultures. The 18 S mRNA levels were normalized to β-actin mRNA levels in the same sample.

**PCR Turnover Assay**—Basal and agonist-mediated βAR turnover were determined by following the loss of surface-biotinylated receptors. Briefly, the cultures grown in 35-mm dishes were treated with Br-To-ATP (40 nM NaHCO3, 100 mM NaCl, pH 8.6), incubated with 1 μM sulfo-NHS-LC-biotin in Buffer B at 4 °C for 30 min, and rinsed with ice-cold DPBS, 20 mM glutamine (27). The cells then were warmed to 37 °C in fresh medium in the absence or presence of 10 μM ISO and collected at different times as described above for the total βAR binding assay. Portions (0.5–1 μg) of the lysates were resolved on a 6% Tris/borate/EDTA gel, which was dried and exposed to a Bio-Rad Storage Phosphor Screen. The screen was scanned, and the labeled bands were quantitated using a Bio-Rad Image Master imaging system and multi-analysis/PC software. The abundance of each βAR mRNA was calculated relative to that of the control 18 S rRNA band from the same reaction.

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**Materials and Methods**—The materials and methods used in this study were the same as for the βAR binding assay.
subjected to SDS-PAGE and blotting with HRP-conjugated streptavidin as described below.

Whole Cell Phosphorylation of βARs—BHK cells grown in 6-well plates were transfected with one of the pcDNA3 plasmids encoding wild-type or chimeric HA-βARs (1 μg DNA/well; 3 wells per plasmid). After 24 h, one well of each set of three was assayed for total binding activity. The other two wells were washed with serum- and phosphate-free Dulbecco’s modified Eagle’s medium/Hepes, incubated in the same medium containing 200 μCi/ml of [32P]orthophosphate for 2.5 h and then for 10 min ± 1 μM ISO. The cells were washed extensively with ice-cold Ca2+- and Mg2+-free DPBS and lysed in 0.7 ml of radioimmune precipitation assay buffer with protease and phosphatase inhibitors. The lysates were extracted and centrifuged as described above. The soluble extracts were pre-cleared by gently rotating with 30 μl of protein A-Sepharose for 1 h. After removing the beads, the soluble samples were added to tubes containing 5 μl of anti-HA-Sepharose and 25 μl of protein A-Sepharose (added as carrier to have a visible pellet), and the tubes were rotated at 4 °C overnight. The beads were washed five times with 300 μl of radioimmune precipitation assay buffer, and bound proteins were eluted in 50–80 μl of sample buffer (based on lystate binding, adjusted to make βARs/μl the same in samples from control and stimulated cells) as described above. Portions of 40 μl were separated by SDS-PAGE, and the gel was dried and exposed to a phosphor screen for 20 to 30 h. The screen was scanned and analyzed as described above. We confirmed that equal amounts of receptors were immunoprecipitated from control and stimulated cells by transferring proteins from the gel to a blot that was probed with rat anti-HA and HRP-conjugated mouse anti-rat.

Western Blotting—Immunoblotting of βAR proteins was done essentially as described previously (27). Briefly, portions of cell lysates or immunoprecipitates dissolved in SDS sample buffer were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The latter were blocked with casein in Tris-buffered saline, blotted with anti-β1AR (1:10000), anti-β2AR (1:4000), or anti-arrestin (1:4000) antibodies, washed, and blotted with HRP-conjugated goat anti-rabbit or -mouse IgG or streptavidin (1:10000) as appropriate. Then the blots were visualized by enhanced chemiluminescence, and the images were captured on Eastman Kodak Co. Bio-Max MR or Lite 2 film and quantified using NIH Imaging software. βAR produces a linear response between 2.5 and 25 fmol (27). Similar results were observed with β2AR (data not shown).

Data Analysis—Unless otherwise indicated, each experiment was repeated at least three times, and each data point within an experiment was done in triplicate. Data were fitted to curves by nonlinear regression analysis and analyzed for statistical significance by one- or two-way analysis of variance or two-tailed t test using Prism 3 (GraphPad Software).

RESULTS

Opposite Changes in Levels of β1AR and β2AR by Persistent Agonist Stimulation—To investigate the effects of persistent agonist stimulation on β1AR and β2AR, we exposed clonal lines of BHK cells stably expressing either subtype at ~1 pmol/mg protein (BHK-hβ1 and hβ2 cells) to 10 μM ISO for up to 24 h. The cells then were lysed and assayed for total βAR binding activity using the hydrophobic radioligand [125I]CYP. The number of β1AR binding sites decreased to 52% of control with a t½ of 2.6 h whereas that of β2AR increased to 131% of control (Fig. 1A). Although there was an initial, modest reduction of β2AR binding, overall β2AR underwent up-regulation in contrast to the rapid down-regulation of β1AR. To determine whether the changes in binding activity reflected corresponding changes in βAR proteins, we subjected the same samples to Western blotting with antibodies to βAR C-terminal sequences. With increasing time of agonist treatment, the levels of immune-detected β1AR increased, and those of β2AR decreased (Fig. 1, B and C), establishing that the respective up- and down-regulation of binding activities occurred at the receptor protein level. β2AR binding activity initially decreased more rapidly than the immune-detected receptor proteins (see Fig. 1 and Table 1). A similar delay was observed in the down-regulation of HA-β2AR expressed in mouse 1-cells (13). In general, our results are consistent with previous studies that β2AR is more resistant to agonist-mediated down-regulation than β1AR.

FIG. 1. Persistent agonist-mediated regulation of βAR subtypes expressed in BHK cells. BHK cells stably expressing ~1 pmol of β1AR (●) or β2AR (■) per mg of protein were exposed to 10 μM ISO for the indicated times, washed, and lysed. The whole cell lysates were then assayed for the total number of βAR binding sites with [125I]CYP (A) and for receptor protein by Western blotting with anti-βAR antibodies (B) and quantification of the immunoblots by densitometric analysis (C) as described under “Experimental Procedures.” Data are the mean ± S.E. of three-six independent experiments.

The changes in β1AR and β2AR binding activity after 24 h was dependent on agonist concentration with half-maximal effects occurring at ~1–3 nM ISO (Fig. 2A). When we compared short-term and prolonged agonist stimulation on βAR regulation, we found that a 15-min exposure had only a slight immediate effect on total βAR binding activity but led to changes 24 h later, especially for β1AR (Fig. 2B). β1AR levels after 15 min were 103 ± 2% of control and increased to 126 ± 3% after 24 h. β2AR levels decreased to 92.8 ± 9 and 79.6 ± 1% of control after 15 min and 24 h, respectively. Thus, the up-regulation of β1AR after 15 min of agonist stimulation followed by 24 h without agonist treatment was substantial when compared with the up-regulation obtained with constant agonist stimulation for 24 h. In contrast, the down-regulation of β2AR over the same time period was small in comparison to the maximum caused by prolonged stimulation. As cAMP has been reported...
Kinetic parameters of acute and persistent agonist-mediated regulation of β1AR and β2AR expressed in BHK cells

Clonal BHK and Arr2-BHK cells stably expressing β1AR and β2AR were exposed to ISO for up to 30 min and assayed for internalization of cell surface binding sites with [3H]ICYP or for as long as 24 h, lysed, and assayed for total cellular binding sites with [3H]ICYP or analyzed by Western blotting for amounts of immune-detected receptors as described under "Experimental Procedures." Cells also were biotinylated, incubated in the absence and presence of ISO for up to 24 h, and analyzed for amounts of biotinylated receptors remaining by immunoprecipitation and streptavidin overlay. Data were best fit by nonlinear regression analysis to one-phase exponential decay curves by Prism 3, and rates and extents of change are expressed as t1/2 and maximal % change. When the data could not be curve-fitted, the value at 24 h is given. All values are the mean ± S.E. of three to six independent experiments.

| Cells expressing | Total βAR binding sites | Immune-detected βAR protein | βAR turnover | Internalization of surface βAR |
|------------------|-------------------------|----------------------------|--------------|-------------------------------|
|                  | t1/2 (h) | % of con | t1/2 (h) | % of con | t1/2 (h) | % of con | t1/2 (min) | % max |
| β1AR             | 129 ± 9.2 | 51.9 ± 5.7 | 4.4 ± 0.6 | 38.3 ± 3.9 | 6.87 ± 1.8 | 2.97 ± 0.35 | 1.59 ± 0.1 | 51.8 ± 1.8 |
| β2AR             | 107 ± 7.1 | 57.7 ± 3.8 | 6.54 ± 0.7 | 5.00 ± 0.6 | 3.18 ± 0.6 | 15.6 ± 1.3 | 2.87 ± 0.3 | 37.8 ± 1.6 |
| β1AR/Arr2        | 7.4 ± 0.4 | 129 ± 9.2 | 4.4 ± 0.6 | 38.3 ± 3.9 | 6.87 ± 1.8 | 2.97 ± 0.35 | 1.59 ± 0.1 | 51.8 ± 1.8 |

to mediate down-regulation in some cells heterologously expressing βARs (15, 27), we exposed the cells to a permeable cAMP derivative, (chlorophenylthio)-cAMP, for 24 h. The effect was an up-regulation of β2AR (129 ± 1% of control; p < 0.001, n = 3) similar to that induced by agonist. There was no β1AR down-regulation but instead a small, not quite significant up-regulation (116 ± 7.5% of control; p = 0.07). Thus up-regulation of β1AR levels may be mediated by agonist-generated cAMP whereas down-regulation of β2AR may be because of agonist binding.

Agonist-mediated Up-regulation of βAR mRNA—To find out whether changes in βAR mRNA levels contributed to βAR regulation by agonist, BHK-hβ1 and -hβ2 cells were treated with 10 μM ISO over a 24-h period, and β1AR and β2AR mRNA levels were quantified by reverse transcriptase-PCR. As shown in Fig. 3, β1AR mRNA levels increased 3-fold within 1 h of ISO stimulation and then decreased but remained above control levels to the end of the 24-h treatment. β2AR mRNA levels also increased but to a lesser degree. Similar results were obtained with a second clonal line expressing β1AR or β2AR and also co-expressing arrestin-2 (see below).

Prolonged β1AR Regulation Unaffected by Increased Internalization—β1AR is more resistant to agonist-mediated internalization compared with β2AR in CHW (25, 26, 31) and HEK 293 cells (28, 32) as endocytosis of some GPCRs is necessary for their down-regulation (6), including β1AR (27) and β2AR (7, 8), we explored the possibility that subtype differences in internalization might account for differences in down-regulation in BHK cells. Exposing BHK-hβ1 and -hβ2 cells to ISO over a 30-min period resulted in time-dependent internalization of both subtypes with similar first order kinetics (t1/2 values of 3.2 and 2.9 min; see Table I). The maximal internalization of β1AR, however, was only 16% in contrast to 38% of β2AR (Fig. 4A). When the cells were continuously exposed to 10 μM ISO for longer times, the proportion of β1AR remaining on the cell surface actually began to increase, and the difference with β2AR reached 6-fold at 24 h (Fig. 4B).

To investigate whether increasing β1AR internalization would overcome its resistance to down-regulation, we co-expressed arrestin-2 with each β-subtype in BHK cells, which contain low levels of arrestin-3 and no arrestin-2 (38). Arrestins are necessary for endocytosis of GPCRs via clathrin-coated pits (39, 40). We obtained clones that co-expressed ~3 pmol of arrestin-2 and ~1.3 pmol of either β1AR or β2AR per mg of protein (Arr2-BHK-hβ1 and -hβ2 cells) (Fig. 5A). The internalization of β1AR was substantially increased to 52% and that of β2AR was increased to 64% in these cells (Fig. 5B) with a reduction in the t1/2 values to 1.5 min (Table I). When we exposed Arr2-BHK-hβ1 and -hβ2 cells to persistent agonist treatment, β1AR still underwent up-regulation even though 70% or more of the total β1AR was internalized at any given time (Fig. 5C).

The extent of β2AR down-regulation was similar to that in cells not co-transfected with arrestin-2, but rate was significantly slowed (see Table I). In addition, because total β1AR levels increased and β2AR levels decreased, the relative amounts of internalized β1AR were more than those of β2AR, reaching almost 4-fold by 24 h. Thus despite the extensive intracellular accumulation of β1AR, it remained resistant to agonist-mediated down-regulation.

Basal and Agonist-mediated Turnover of β1AR and β2AR—In the absence of agonist stimulation, receptors are

2 W. Liang, S. Austin, Q. Hoang, and P. H. Fishman, unpublished observations.
maintained at a steady state level, the rate of receptor synthesis balanced by receptor turnover (14, 41). We used a biotinylation procedure to identify any differences in either basal or agonist-mediated turnover of $\beta_1$AR and $\beta_2$AR. The cells were labeled with a non-permeable, non-cleavable biotin derivative, incubated up to 24 h in the presence or absence of agonist, and...
solubilized. βARs in the soluble extracts were immunoprecipitated with anti-βAR antibodies, and biotinylated βARs were detected by blotting with HRP-conjugated streptavidin. As shown in Fig. 6 and Table I, basal turnover of both β1AR and β2AR occurred with similar kinetics (t1/2 = 6.5 ± 0.7 and 6.9 ± 1.8 h, respectively), and over 90% of the biotinylated βAR disappeared after 24 h. Whereas agonist stimulation increased βAR turnover significantly (t1/2 = 3.0 ± 0.2 h; p < 0.01), it had only a slight impact on β2AR turnover (t1/2 = 5.0 ± 0.5 h; p > 0.05). We also compared basal and agonist-mediated turnover in the Arr2-BHK-hβ1 and -hβ2 cells and found a similar pattern for the two subtypes (data not shown). Together these results are indicative that βAR is resistant to agonist-mediated degradation in BHK cells, even under conditions in which it undergoes extensive internalization.

To validate the biotinylation method, we used another approach to measure the basal turnover rate of each subtype. For β2AR, we applied the methods and equations described by Williams et al. (14) to the down-regulation data and obtained a t1/2 of 7.2 h for basal turnover. As β2AR did not down-regulate, we took advantage of the receptor being under control of the metallothionein promoter, exposed BHK-hβ1 cells to zinc sulfate to induce more receptors, and followed their turnover as described by Dunigan et al. (27). A t1/2 of 7.2 h for the turnover of zinc-induced β2AR in BHK cells was obtained. These values are very close to the basal turnover rates obtained using biotinylation assay, indicating that biotinylation did not alter the turnover of either subtype.

**Effects of Blocking Trafficking to Lysosomes on Basal and Agonist-mediated Turnover of β-Subtypes**—Little is known about basal turnover of GPCRs, and although it is general accepted that agonist-mediated degradation occurs in lysosomes (6), non-lysosomal proteolysis of β2AR has been described (13). To investigate the two possibilities, we used bafilomycin A1, which has been shown to block trafficking of β2AR from endosomes to lysosomes (8). Biotin-labeled BHK-hβ2 and -hβ3 cells were incubated in the presence and absence of 1 μm bafilomycin A1 and/or 10 μM ISO and were analyzed for the disappearance of biotinylated βAR after 4 h. Bafilomycin A1 slightly increased the basal turnover of β1AR and β2AR but effectively blocked the agonist-mediated turnover of β2AR (Fig. 7). Based on these results, it appears that the basal degradation of both subtypes may be non-lysosomal, whereas agonist-mediated degradation of β2AR is lysosomal.

**Role of the C-tail of βAR in Agonist-mediated Regulation**—As the C-tails of a number of GPCRs have been identified as structural determinants in targeting the receptors to lysosomes (33–35), we explored the effect of replacing the C-tail of one subtype with that of the other on agonist-mediated regulation. As the first step in the regulatory process is receptor phosphorylation, we 32P-labeled BHK cells transiently expressing β1AR, β2AR, or the chimeras, β1/β2ct-AR and β2/β1ct-AR, stimulated the cells with agonist, and purified and analyzed the receptors for 32P incorporation (Fig. 8). Whereas agonist stimulation increased the phosphorylation of β2AR and β1/β2ct-AR by 271 and 200% of control, respectively, it had no significant effect on the phosphorylation of β1AR and β2/β1ct-AR. We next compared the agonist-mediated internalization of the wild-type and chimeric receptors (Fig. 9A). β1/β2ct-AR underwent more internalization than wild-type β1AR, and β2/β1ct-AR underwent less than wild-type β2AR, which is in agreement with previous findings in HEK 293 cells (32). Having confirmed that the chimeras behaved similarly in BHK cells, we investigated the effects of persistent agonist stimulation on their regulation. As shown in Fig. 9B, β1/β2ct-AR underwent down-regulated and thus was more similar to β2AR, the source of its C-tail, than to β1AR, which represented most of its structure. In an analogous manner, β2/β1ct-AR exhibited up-regulation, the same as β1AR from which it derived its C-tail. Western blotting confirmed the down- and up-regulation of the chimeras occurred at the protein level (Fig. 9C). Although the magnitude and rate of up- or down-regulation of each chimera were respectively less and slower than the corresponding wild-type βAR source of its C-tail, the results clearly demonstrated that the C-tails are important determinants not only of rapid agonist-mediated phosphorylation and internalization but also of persistent agonist-mediated up- or down-regulation of β1AR and β2AR.

It was noted that although the β1/β2ct-AR expression vector contains the 3′-untranslated region of the β2AR gene, the β2/β1ct-AR expression vector lacks the 3′-untranslated region of the β1AR gene. Thus, the mRNA of the β2/β1ct-AR chimera does not have a 3′-untranslated region, which often contains specific sequences recognized by mRNA-binding proteins that modu-
late mRNA stability. Therefore, we determined the mRNA levels of the chimeras. A 24-h exposure to 10 μM ISO did not change the levels of βAR/ct-AR mRNA (98.5 ± 1.2% of control) and slightly but significantly increased the levels of βAR/ct-AR mRNA (116 ± 1.1% of control) (Fig. 3). Apparently, the up-regulation of βAR mRNAs in BHK cells is not dependent on the 3'-untranslated region.

To further confirm the role of the C-tail on βAR regulation, we determined the turnover of the chimeric receptors. We labeled BHK cells stably expressing wild-type and chimeric βARs with [32P]-labeled, incubated ± 1 μM ISO for 10 min, washed, lysed, and solubilized. Receptors were absorbed to anti-HA-Sepharose, eluted, and separated by SDS-PAGE.

**Fig. 8. Phosphorylation of wild-type and chimeric βARs in response to agonist stimulation.** A, BHK cells transiently expressing wild-type or chimeric HA-βARs were [32P]-labeled, incubated ± 1 μM ISO for 10 min, washed, lysed, and solubilized. Receptors were absorbed to anti-HA-Sepharose, eluted, and separated by SDS-PAGE. Molecular mass markers are indicated in kDa. B, shown is the quantification of receptor phosphorylation expressed as % of control. Data are the mean ± S.E. of three-four independent experiments. ns, p > 0.05; ***, p < 0.001 (one-way analysis of variance with Bonferroni’s post test).

In the present study, we have shown that in response to prolonged stimulation by agonist, βAR underwent up-regulation in contrast to the rapid down-regulation of βAR in BHK cells. The changes in receptor binding activity were accompanied by corresponding changes in immune-detected receptor proteins. Thus, up- and down-regulation occurred not by activating or inactivating pre-existing receptors but by varying the rates of receptor synthesis or turnover. To resolve how the two processes contribute to this subtype difference, we determined both the levels of βAR mRNA and the kinetics of βAR degradation. Our results showed that in agonist-treated cells, the mRNA levels of both subtypes were increased, being greater for β2AR. Although agonist-induced decreases in levels of both human βAR and β2AR mRNA have been observed in CHW cells (15, 27), up-regulation of the mRNA levels of other GPCRs has been reported, an example being the dopamine D2L receptor stably expressed in Chinese hamster ovary cells (42). The basal turnover rate as measured by the disappearance of biotin-labeled βAR was essentially the same for both subtypes. Although the degradation of βAR was significantly accelerated by agonist stimulation, βAR turnover was not. In cells treated with a permeable cAMP derivative, up-regulation of β2AR was similar to that with agonist treatment whereas down-regulation of βAR was not observed. A 15-min pulse with agonist caused a substantial up-regulation of β2AR in cells washed and incubated without agonist for 24 h. For β2AR, the corresponding down-regulation was much less. This suggested that the brief stimulation of β2AR produced a signal or activated a pathway in the cells that did not require the further presence of agonist. Based on these results, we propose that the up-regulation of β2AR by prolonged agonist stimulation is because of a CAMP-mediated increase in receptor mRNA levels and the resistance to agonist-mediated degradation, and the down-regulation of βAR is because of the agonist-mediated increased rate of turnover.

We should note that the rates of basal turnover of both β-subtypes and of agonist-mediated turnover of β2AR are relatively rapid. Many previous studies indicated that in the absence of agonist, βARs turn over very slowly with a turnover half-time of 30–200 h (41). However, the t1/2 for the basal turnover of endogenous β2AR and βAR in rat C6 glioma cells are 6.4 and 9.4 h, respectively (43), and 11 h for endogenous β2AR in mouse S49 lymphoma cells (44). Regarding the agonist-mediated turnover of β2AR in BHK cells, the down-regulation of receptor activity occurred with a t1/2 of 2.3 h, which is consistent with other reports on β2AR. Examples are as follows: L cells, –5 h; A431 cells, –1 h (13); BEAS-2B cells, 3.3 h (14); and CHW cells, 2.6–7.3 h (15, 26, 27). When making such comparisons, one must keep in mind that whereas basal turnover is independent of receptor density, agonist-mediated turnover is sensitive to receptor density (27).

We also established that bafilomycin A1, which disrupts trafficking of β2AR from endosomes to lysosomes (8), blocked the agonist-mediated degradation of β2AR but not the basal turnover of either subtype. Together with the similar basal turnover rates, it is reasonable to assume that both subtypes are turned over by the same process. Both human β1AR and β2AR have been located in microdomains of the plasma membrane known as caveolae (45), and similar results were obtained with BHK-hβ1 and -hβ2 cells.3 Basal turnover of βARs may involve a caveolae-associated process occurring either within the caveolae or after caveolae-mediated endocytosis (46). Although the mechanism(s) and pathway by which βARs undergo basal turnover are not known, our results suggest that agonist-mediated turnover is a separate process and not just an increase in the rate of the basal process.

More than one mechanism has been proposed for the down-regulation of β2AR (6). Upon agonist stimulation, β2AR is internalized through clathrin-coated pits and undergoes endosomal trafficking and sorting either to the recycling pathway or to the late endosome/lysoosome pathway (7, 8). Internalization is necessary for lysosomal degradation in this model. Other studies using mutagenesis or inhibitors of endocytosis showed

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3 J. Shor, P. K. Curran, and P. H. Fishman, unpublished observations.
that blocking of either internalization (10, 11, 13) or down-regulation (9, 12, 47) of β2AR does not prevent the other process from occurring. In this model distinct pathways are used for internalization and down-regulation of β2AR, and degradation may be independent of internalization, as well as non-lysosomal. We found that in BHK cells, overexpression of arrestin-2 increased the extent of internalization but not down-regulation of β2AR. This does not eliminate the possibility that β2AR internalization is necessary for its down-regulation. In fact, the inhibition of agonist-stimulated turnover of β2AR by bafilomycin A1 is consistent with this possibility. Although β2AR undergoes less agonist-mediated internalization than β1AR, increasing β2AR internalization by overexpressing arrestin-2 did not result in its down-regulation. β2AR continued to undergo up-regulation in agonist-treated cells even though most of the receptors remained internalized. Thus, differences in subtype internalization did not contribute to differences in their down-regulation.

There is increasing evidence the C-tails of GPCRs are important determinants in regulating their endosomal trafficking (33–36). For the C-tail of β2AR, tyrosine 350 and 354 are required for receptor down-regulation (9), and the terminal four amino acid residues (DSSL) represent a PDZ domain binding motif that is involved in the sorting of β2AR between recycling endosomes and lysosomes (34, 36). β1AR has no tyrosine residues in its C-tail but does terminate in a PDZ binding motif (ESKV) that is recognized by PSD-95 and MAGI-2, two postsynaptic scaffolding proteins that respectively inhibit and enhance receptor internalization (48, 49). Mutation of the PDZ motif of mouse β1AR enhances agonist-mediated endocytosis in mouse cardiac myocytes (50). When we used chimeric receptors in which the C-tails had been exchanged, we found that upon persistent agonist stimulation, β1/β2ct-AR underwent up-regulation, and β1/β2ct-AR underwent down-regulation, the order of down-regulation being β2AR > β1/β2ct-AR > β1ct-AR > β1AR. The same order was observed for agonist-mediated turnover of biotinylated receptors, as well as internalization of cell surface receptors. The C-tails also contribute to the differences in agonist-mediated desensitization of the two subtypes (51) and thus are involved in all the major mechanisms of βAR regulation. Furthermore, we demonstrated that agonist-stimulated phosphorylation of the receptors was determined by the C-tails as an increase occurred in β2AR and β1/β2ct-AR but not in β1AR and β1/β2ct-AR. The agonist-stimulated increase in phosphorylation of β1AR also is much less than that of β2AR in HEK 293 cells (52). These differences in subtype phosphorylation may account for the differences in other downstream regulatory events such as binding of arrestins, arrestin-mediated desensitization and internalization, and possibly down-regulation.

As up- or down-regulation of the chimeras is not as effective as that of the wild-type receptors, other receptor domains may contribute to their regulation. One candidate is the proline-rich region in the third intracellular loop of β2AR that is recognized by Src homology 3 domain-containing proteins of the endophilin family (53). Overexpression of endophilin (53) or deletion of the region (31) enhances β2AR internalization. Another candidate is the N-terminal region that contains an allelic polymorphism at codon 49. When expressed in HEK 293 cells, the less frequent Gly-49 variant of the human β2AR undergoes some agonist-mediated down-regulation whereas the more abundant Ser-49 variant is resistant and even exhibits up-regulation (29, 30).

The cell type-specific effects on β1AR regulation are very striking. In both BHK and HEK 293 cells, β1AR is totally resistant to agonist-mediated down-regulation. In contrast, we have shown previously that β1AR endogenously expressed in SK-N-MC cells (54) or stably expressed in CHW cells (27) undergoes agonist-mediated down-regulation. This apparent anomaly is most likely explained by the existence of sorting
proteins that recognize specific motifs on receptors and control their endosomal trafficking. The PDZ binding motif in the C-tail of β2AR interacts with PDZ domain-containing proteins of the Na+/H+ exchanger regulatory family (34). When the motif is mutated or phosphorylated by G protein-coupled receptor kinase 5, the interaction is disrupted, and receptor trafficking is shifted from recycling to degradation. More recently, a cytosolic protein named GASP (GPCR-associated sorting protein) has been identified that binds to the C-tails of a subset of GPCRs including β2AR and facilitates receptor trafficking to lysosomes (55). Sorting nexin 1, a membrane-associated protein, also mediates GPCR sorting to lysosomes (56). Most likely, sorting proteins that recognize the C-tail of β2AR exist, and their levels of expression vary among different cell types. Such variations have been observed for G protein-coupled receptor kinases and arrestins (38, 57). In the absence of a β2AR-selective sorting protein, the β2-subtype may only be recycled. A further consideration is that differences in posttranslational receptor modifications such as phosphorylation (present study and Ref. 52) and ubiquitination (58) may contribute to cell type, as well as subtype, dissimilarities in receptor degradation.

Finally, the combination of limited internalization and up-regulation of β2AR in contrast to more extensive internalization, and down-regulation of β2AR results in a large disparity between the subtypes in the number of cell surface receptors present after agonist stimulation for 24 h. Whereas β2AR levels were less than 20% of control, β1AR levels were more than control. As β2AR has been found to undergo less agonist-mediated desensitization than β2AR (26, 51), β1AR may remain responsive to agonist stimulation for prolonged periods. This capacity may be useful in certain cell types but not in others such as cardiomyocytes where the apoptotic effects of β2AR could be detrimental. Our identification of the receptor C-tail as an important structural element in determining subtype down-regulation may provide a basis for future studies and therapeutic strategies.

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