Complexity of Agonist- and Cyclic AMP-Mediated Downregulation of the Human β_1-Adrenergic Receptor: Role of Internalization, Degradation, and mRNA Destabilization

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ABSTRACT: Prolonged agonist exposure often induces downregulation of G protein-coupled receptors (GPCRs). Although downregulation of the prototypical β_2-adrenergic receptor (β_2AR) has been extensively studied, the underlying mechanisms have yet to be resolved. As even less is known about the β_1-subtype, we investigated the downregulation of human β_1AR stably expressed in Chinese hamster fibroblasts in response to the agonist isoproterenol or the cell-permeable, chlorophenylthio-cAMP (CPT-cAMP). While either effector mediated decreases in both β_1AR binding activity and steady-state β_1AR mRNA levels, there were significant differences in their actions. Whereas agonist-mediated downregulation of β_1AR followed first-order kinetics, that induced by CPT-cAMP was delayed for several hours and ~50% of the former. Furthermore, agonist but not CPT-cAMP induced β_1AR internalization, and inhibiting internalization also suppressed agonist-mediated downregulation. The latter, however, was more sensitive than the former to agonist concentration (EC_{50} of 0.3 vs 48 nM). Thus, at ≤1 nM agonist, downregulation occurred without internalization and with a pattern similar to that mediated by CPT-cAMP. The amounts of β_1AR downregulated or internalized were proportional to initial receptor levels but reached saturation at ~2 and 3 pmol/mg of protein, respectively. The fate of β_1AR protein during downregulation was determined by immunoblotting with anti-C-terminal antibodies. In agonist-treated cells, β_1AR protein disappeared with time and without any immunoreactive degradation products. Agonist-mediated downregulation of the human β_1AR appears to be a complex process that consists of both agonist- and cAMP-specific components. The former involves both receptor internalization and degradation whereas the latter involves a reduction in receptor mRNA.

The human β_2-adrenergic receptor (β_2AR)[1] has been the prototype for elucidating mechanisms of regulation of GPCRs (*1*). Following agonist stimulation, β_2AR undergoes desensitization, internalization, and downregulation. Although the three regulatory processes are temporally and mechanistically distinct, they all serve to attenuate receptor signaling. Desensitization is initiated by phosphorylation of β_2AR by protein kinase A (PKA) and GPCR kinases (GRKs). Phosphorylation by the latter facilitates β-arrestin binding to the receptor which impairs its coupling to G_s (*2*). β-Arrestin also mediates β_2AR internalization by functioning as an adaptor protein between the receptor and clathrin and directing the receptor to the clathrin-coated endocytosis pathway (*3*, *4*). Downregulation of β_2AR occurs after more prolonged agonist exposure and operationally has been defined as a loss of total cellular binding activity as most studies do not distinguish between receptor inactivation and degradation. The fate of the receptor protein, however, remains unclear (*5*, *6*) as does the relationship between endocytosis and downregulation of β_2AR (*7*). Using confocal fluorescence microscopy, both green fluorescent protein- and epitope-tagged β_2AR have been observed to slowly accumulate in lysosomes (*8*−*10*). Blocking β_2AR endocytosis in COS-1, HeLa, and HEK 293 cells inhibits its downregulation (*9*) whereas in murine L and human A431 cells, β_2AR downregulation still occurs (*11*). More recently, a kinetic approach was used to develop a two-component model for downregulation of β_2AR: a low-affinity, high-capacity component associated with internalization and a high-affinity, low-capacity component independent of internalization (*12*). Finally, a reduction in steady-state β_2AR mRNA levels occurs in some cells either by destabilization of β_2AR mRNA (*13*−*15*) or by repression of β_2AR gene transcription (*16*). Both are mediated by cAMP and contribute to the downregulation of β_2AR.

Less is known about the regulation of the human β_1AR even though it is the major subtype in heart, adipose tissue, and certain brain regions (*17*). Several studies have found that the human β_1AR is more resistant than the human β_2-

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[1] Abbreviations: βAR, β-adrenergic receptor; β_1AR, β_1-subtype; β_2-AR, β_2-subtype; CPT-cAMP, (chlorophenylthio)adenosine 3′,5′-monophosphate; CHW, Chinese hamster fibroblast; ConA, concanavalin A; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA-β_2AR, hemagglutinin-tagged β_2AR; ^{125}ICYP, (−)-[^{125}I]iodocyanopindolol; ISO, isoproterenol; PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; TBSE, Tris-buffered saline/EDTA.

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AR to agonist-mediated regulation including downregulation (*18*−*22*). In the present study, we investigated the downregulation of the human β_1AR stably expressed in Chinese hamster fibroblast (CHW) cells in response to both agonist and a cell-permeable cAMP derivative. We not only determined the changes in binding activity in cells expressing a wide range of receptor levels but also analyzed changes in β_1AR mRNA and β_1AR protein, the latter by immunoblotting with antibodies to the C-terminal region of the wild-type receptor. Our results indicate that agonist-mediated downregulation of human β_1AR is complex, involves different mechanisms than cAMP-mediated downregulation, and yet consists of both agonist- and cAMP-specific elements. We show that the agonist-specific component of downregulation requires β_1AR internalization and correlates more closely with receptor degradation while the cAMP-specific component does not require internalization and is more closely associated with downregulation of β_1AR mRNA levels.

EXPERIMENTAL PROCEDURES

Materials. (−)-Isoproterenol (ISO), (−)-propranolol, concanavalin A (ConA), isobutylmethylxanthine, and bovine serum albumin were from Sigma; (chlorophenylthio)adenosine 3′,5′-monophosphate (CPT-cAMP), leupeptin, E-64, soybean trypsin inhibitor, and Pefabloc SC were from Roche; benzamidine and calpain inhibitor I were from Calbiochem. Horseradish peroxidase-conjugated goat anti-rabbit IgG and protein A−agarose were from Zymed Laboratories; rabbit antibodies to human β_1AR (A-20) were from Santa Cruz Biotechnology; casein in Tris-buffered saline blocking solution was from Bio-Fx Laboratories, Owing Mills, MD. BCA protein reagent, SuperSignal West Pico, and Dura Extended chemiluminescent substrates, horseradish peroxidase-conjugated streptavidin, sulfo-NHS-LC-biotin, and biotin-LC-hydrazide were from Pierce. (−)-[^{3}H]CGP-12177 (45 Ci/mmol) was from Amersham and (−)-[^{125}I]iodocyanopindolol (2200 Ci/mmol) from NEN. Ambion was the source of reagents for the preparation of RNA and the ribonuclease protection assay.

Cells and Cell Culture. Clonal lines of CHW cells stably expressing the human β_1AR under control of the metallothionein promoter (*20*) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 200 μg/mL G418. To induce higher levels of β_1AR expression, the cells were exposed to medium containing 20 μM EDTA and increasing concentrations of $ZnSO_4$. To induce downregulation, cells were exposed to either 10 μM ISO or 200 μM CPT-cAMP for the times indicated. HEK 293 cells obtained from the American Type Culture Collection were transiently transfected with the plasmids zem228c-hβ_2AR or -hβ_1AR (*20*) using Lipofectamine Plus (Life Technologies). HEK 293 cells stably expressing an epitope-tagged human β_2AR (*23*) were generously provided by Dr. Richard Clark, University of Texas Medical School, Houston, TX.

Receptor Binding Assays. Control and treated cells were washed in situ at 4 °C twice with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) and once with 1 mM Tris-HCl/2 mM EDTA, pH 7.4, and allowed to detach and lyse in the latter solution containing protease inhibitors (10 μg/mL of leupeptin, soybean trypsin inhibitor, and benzamidine, and 1 mM EGTA and Pefabloc SC). Portions were assayed for protein by a microtiter plate assay using BCA reagent and for binding activity as described previously (*20*, *24*). Briefly, samples containing 5−10 fmol of β_1AR were incubated in 250 μL of 50 mM Hepes, pH 7.5, 4 mM $MgCl_2$, 0.04% bovine serum albumin, and 200−250 pM ^{125}ICYP for 1 h at 30 °C. Nonspecific binding was measured by including 10 μM (−)-propranolol. The samples were filtered under vacuum onto glass fiber filters (Schleicher & Schuell no. 32) that were counted in a γ-counter. Receptor internalization was assayed as described previously (*20*, *24*). Briefly, cells grown in 24-well plates were exposed to ISO or CPT-cAMP in DMEM/25 mM Hepes at 37 °C for the times indicated, washed with ice-cold DPBS, and incubated with 5 nM (−)-[^{3}H]CGP-12177 in Eagle's minimal essential medium/25 mM Hepes/30 μg/mL bovine serum albumin for 1 h at 4 °C with and without 10 μM (−)-propranolol. The cells were washed with ice-cold DPBS and dissolved in 250 μL of 1 M NaOH; 25 μL portions were assayed for protein, and the remaining portions were neutralized with glacial acetic acid and assayed for ^{3}H by liquid scintillation counting. To block internalization, cells were incubated in medium containing 0.45 M sucrose or serum-free medium containing 0.25 mg/mL ConA for 30 min. The latter was replaced with medium and ConA, and the cells were exposed to agonist for different times. To measure cAMP responses, control and treated cells in 24-well plates were incubated at 37 °C in DMEM/25 mM Hepes/1 mM isobutylmethylxanthine for 10 min, stimulated with 1 μM ISO for 10 min, and assayed for cAMP by radioimmune assay (*25*).

Separation of Plasma and Endosomal Membranes. Heavy and light membrane fractions were prepared by differential centrifugation using a modification of the procedure described by Jockers et al. (*11*). After the cells were washed and lysed as described above, the lysate was centrifuged at 450*g* for 5 min. The resulting postnuclear supernatant was layered on top of a 1 mL cushion of 35% sucrose and centrifuged at 150000*g* for 90 min at 4 °C in a Beckman SW 55Ti rotor. The layer of light membranes at the interface and the heavy membrane pellet at the bottom of the cushion were collected, diluted up to 5 mL in lysis buffer, and centrifuged at 200000*g* for 60 min at 4 °C. The membrane pellets were diluted in 0.5 mL of lysis buffer, and portions were assayed for binding and subjected to Western blotting analysis.

Western Blotting. Antibodies were raised against a peptide, AADSDSSLDEPCRPGFASES, corresponding to the C-terminus (Ala^{456}-Ser^{475}) of the human β_1AR (*26*). Some of the peptide was amidated on the C-terminal carboxyl group. Both peptides were coupled to keyhole limpet hemocyanin by the glutaraldehyde method, and rabbits were immunized with the resulting antigens using standard procedures (*27*). Sera were assayed for antipeptide antibodies by ELISA, and antisera exhibiting high titers to the amidated peptide (PF11, 1:10000) and the nonamidated peptide (PF13, 1:3000) were used for immunoblotting. A commercial antibody (A-20) raised against the nonamidated peptide also was used. Cell lysates were mixed with 4× SDS sample buffer (3:1 v/v), dissolved at 42 °C for 45 min, and separated by 10% SDS−PAGE. The proteins were transferred to poly(vinylidene difluoride) membranes (Millipore Immobilon-P), which were blocked with 1% blocking reagent, and blotted in 0.5%

blocking reagent with anti-β_1AR antiserum (usually PF11 at 1:10000) overnight at 4 °C and then with the secondary antibody (1:10000) for 30 min at 25 °C. Between incubations, they were washed with Tris-buffered saline/0.1% Tween 20. After the blots were incubated in chemiluminescent substrate and exposed to Kodak Bio-Max MR or Light film, the images were quantified using a LaCie Silverscanner III and NIH Image software, version 1.61. To ensure that the exposure levels were within the dynamic range of the film, we usually loaded additional amounts of the control lysate (120% and 50% of the normal amount) on the gel. In addition, we used a step tablet (Kodak T-27) to check the linearity of the scanner and software analysis and observed that the measured values increased almost linearly between 0 and 2.00 OD.

Surface Biotinylation and Immunoprecipitation of β_1AR. Cell monolayers were rinsed with ice-cold DPBS and with 40 mM sodium bicarbonate buffer, pH 8.6/100 mM NaCl and incubated in the same buffer containing 1 mM sulfo-NHS-LC-biotin for 30 min at 4 °C to label amino groups, or incubated in DPBS/20 mM $NaIO_4$ for 30 min at 4 °C in the dark, rinsed twice with DPBS and once with 100 mM sodium acetate buffer, pH 5.5/1 mM $CaCl_2$/0.5 mM $MgCl_2$, and incubated in the same buffer containing 2 mM biotin-LC-hydrazide for 30 min at 4 °C to label carbohydrate groups. The cells were washed and lysed as described above, and the lysates were mixed 4:1 (v/v) with 5× RIPA buffer (1× = 1% NP-40/0.5% sodium deoxycholate/0.1% SDS/20 mM Hepes, pH 7.5/100 mM NaCl/5 mM EDTA/2 mM EGTA and protease inhibitors) and extracted for 45 min at 4 °C with gently rotation. After centrifugation at 14000*g* for 20 min, the soluble extracts were immunoprecipitated as follows: 50 μL portions of a 50% slurry of protein A−agarose were washed with TBSE buffer (20 mM Tris-HCl, pH 7.4/100 mM NaCl/5 mM EDTA) and then rotated with 20 μL of antiserum PF-11 diluted with 180 μL of TBSE buffer for 1 h at 4 °C. After the beads were washed twice with TBSE buffer and once with RIPA buffer, the soluble cell extracts were added. The samples were rotated for 2 h at 4 °C, and then the beads were washed three times with RIPA buffer and eluted with SDS sample buffer. The eluted material was subjected to SDS−PAGE, transferred to membranes, blotted with horseradish peroxidase−streptavidin (1:10000), and detected by chemiluminescence.

Riboprobe Preparation. A 545 bp fragment of the human β_1AR cDNA (+351 to +896) was isolated by *Pst*I/*Not*I digestion, cloned into pBluescript KS^+ (Stratagene), and linearized by *Asc*I digestion at a unique site within the hβ_1-AR insert (+701). The Ambion BrightStar BIOTINscript in vitro transcription kit was used to synthesize a 216 bp biotin-labeled hβ_1AR antisense riboprobe. One microgram of the above linearized plasmid was incubated in 20 μL containing 2 μL of 10× buffer, 12.5 units of placental RNase inhibitor, 10 units of T7 polymerase, and 10 μL of 2× biotin-14-CTP/NTP mix for 2 h at 37 °C and then for 15 min with 2 units of DNase I to remove template DNA. The transcript was gel-purified, eluted, and ethanol-precipitated, and its concentration was determined by absorbance at 260 nm and purity by the 260/280 ratio; ~10 μg of biotinylated riboprobe was obtained. To make the sense strand, the β_1AR cDNA fragment was linearized by *Sac*I digestion 3′ to the insert and transcribed using T3 polymerase. Cyclophilin mRNA was used for the internal standard. Biotin-labeled and unlabeled cyclophilin riboprobes were transcribed from pTRI-cyclophilin-rat (Ambion) and were diluted 1:9 to compensate for the higher mRNA abundance. In some of the initial experiments, 18S rRNA or β-actin mRNA was used as the internal standard.

Ribonuclease Protection Assays. Cells in 25 cm^2 flasks (~5×10^6 cells) were washed twice with ice-cold DPBS, detached in DPBS/2 mM EGTA/2 mM EDTA, collected by centrifuging at 450*g* for 5 min, and suspended at 10^7 cells/mL in Lysis/Denaturation solution (Ambion Direct Protect kit). Portions equal to $(2.5-4.8) \times 10^5$ cells were hybridized to 500 pg each of the biotinylated β_1AR and cyclophilin riboprobes, and increasing amounts of β_1AR sense strand were hybridized to the β_1AR riboprobe for quantification. After overnight hybridization at 45 °C, single-stranded RNA was digested at 37 °C for 30 min each with a mixture of RNase A (5 units) and T1 (200 units) and then with 100 μg of proteinase K in 0.4% sodium sarcosyl. The protected RNA was ethanol-precipitated, suspended in 10 μL of gel loading buffer, denatured at 95 °C for 4 min, and separated on a 6% polyacrylamide/8 M urea gel for 30 min at 250 V. The samples were transferred onto positively charged nylon membranes by electroblotting at 300 mA for 1 h and cross-linked to the membranes at 1200 μJ using the UV Stratalinker 1800. Samples were detected by the Ambion BrightStar BioDetect kit and quantified by densitometry as described above. The amount of β_1AR mRNA was corrected for the amount of cyclophilin mRNA in the sample and then expressed as percent of control. β_1AR mRNA stability was determined by actinomycin D treatment (2 μg/mL) as described previously (*16*).

Data Analysis. Unless otherwise indicated, each experiment was done at least three times, and the results are given as the means ± SE. Means were compared for statistical significance using a two-tailed *t* test. Within each experiment, each data point was assayed in triplicate and is presented as the mean ± SD. The downregulation data were fitted to a one-phase exponential decay equation by least-squares, nonlinear regression analysis using Prism Version 3.0 (GraphPad Software, San Diego, CA):

$$R = (R_0 - R_f)e^{-k_{dr}t} + R_f \quad (1)$$

where R is the receptor level at time t, R_0 and R_f the initial and final levels, and k_{dr} the first-order rate constant for downregulation. The following equations were used to determine β_1AR basal metabolism (*28*):

$$R = \frac{k_s}{k_{bsl}}(1 - e^{-k_{bsl}t}) + R_0 e^{-k_{bsl}t} \quad (2a)$$

where k_s is the zero-order rate of receptor synthesis and k_{bsl} is the first-order rate constant for receptor turnover. At steady state, the receptor level R_{ss} is a balance between synthesis and turnover:

$$R_{ss} = \frac{k_s}{k_{bsl}} \quad (2b)$$

and one can directly measure either constant and derive the other. We directly measured k_s by inducing cells with $ZnSO_4$

or inactivating β_1AR with 200 μM *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (*29*) and determining the increase or recovery in binding activity with time. To determine k_{bsl}, we removed the $ZnSO_4$ from induced cells and followed the decrease in binding activity with time. We also used Prism 3 to calculate k_s and k_{bsl} from the downregulation data following the method and equations described by Williams et al. (*12*). Values for k_s can be obtained from the following equations:

$$R = \frac{k_s}{k_{dr}}(1 - e^{-k_{dr}t}) + R_0 e^{-k_{dr}t} \quad (3a)$$

When downregulation is complete and a new steady state is reached, eq 3a reduces to

$$R_f = \frac{k_s}{k_{dr}} \quad (3b)$$

Once k_s has been calculated, the corresponding value for k_{bsl} can be determined as described above.

RESULTS

Downregulation of Human β_1AR Binding Activity. When clonal lines of CHW-hβ_1 cells expressing over a 10-fold range of β_1AR levels were exposed to ISO or CPT-cAMP, a time-dependent loss of binding activity was observed (Figure 1). Agonist-mediated downregulation was the more effective, and its kinetics best fit a first-order decay curve (Figure 1A−C). In contrast, that mediated by CPT-cAMP was not first order and exhibited a delay of several hours before any substantial loss was observed. Although cells expressing low β_1AR levels lost their receptors as a percentage more rapidly and extensively than those expressing high levels, downregulation [in pmol (24 h)$^{-1}$ (mg of protein)$^{-1}$] was proportional to receptor density up to ~2 pmol/mg of protein, the highest expression level obtained (Figure 1D). Over this same range, cells exposed to CPT-cAMP lost only half as many receptors in 24 h compared to agonist-treated cells.

Downregulation of β_1AR was also dependent on agonist concentration as is shown in Figure 2 for cells expressing low receptor levels. Both the extent and rate of receptor loss increased with increasing concentrations of ISO (Figure 2A,B), and the half-maximal loss occurred at 0.31 ± 0.1 nM (Figure 2C). This value is substantially lower than the K_d of 31 ± 4.5 nM ($n = 4$) for agonist competition binding to cell membranes in the presence of GTPγS or the EC_{50} of 5.4 ± 0.8 nM ($n = 3$) for agonist-stimulated cAMP formation in the same cells but is sufficient to raise cAMP levels 3.8 ± 0.2-fold ($n = 3$; data not shown). Thus, agonist occupancy alone did not determine the kinetics of β_1AR downregulation, especially at low agonist concentrations. In this regard, the time courses best fit one-phase exponential decay curves for all agonist concentrations except for those of 1 nM or less which displayed a lag of several hours and appeared similar to that mediated by CPT-cAMP (Figure 2B).

As β_1AR expression is under control of the metallothionein promoter (*20*), we induced the cells with increasing concentrations of $ZnSO_4$ to determine the effects of higher receptor levels on downregulation. As levels in low β_1AR-expressing cells increased, the percentage that underwent agonist-mediated downregulation decreased and the cells behaved more like the high-expressing cells (Figure 3). The degree of downregulation became similar (50.4% vs 47.2%) as did the time course ($t_{1/2}$ of 9.4 vs 11.2 h compared to 4.2 h in the noninduced cells). When the high-expressing cells were zinc-induced, β_1AR levels increased to ~7 pmol/mg of protein and the proportion susceptible to downregulation decreased. In agreement with the results in Figure 1D, the number of receptors downregulated was directly proportional to the initial β_1AR density until saturation was reached as receptor expression exceeded 2 pmol/mg of protein (Figure 3, bottom). Saturation of CPT-cAMP-induced downregulation also was observed at these very high β_1AR levels (data not shown).

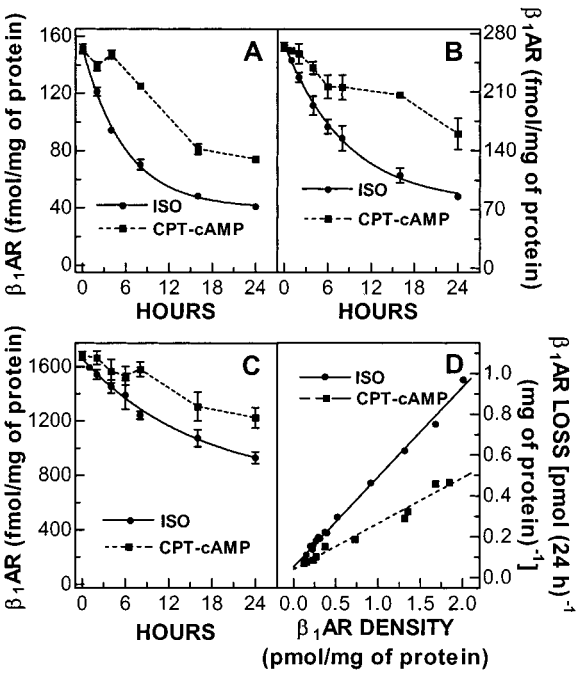

FIGURE 1: Effect of receptor density on agonist- and cAMP-mediated downregulation of human β_1AR in CHW-hβ_1 cells. Cells expressing increasing levels of β_1AR were exposed to 10 μM ISO (●) or 200 μM CPT-cAMP (■) for the indicated times (A−C) or 24 h (D). The cells then were washed, lysed, and assayed for specific ^{125}ICYP binding as described in Experimental Procedures. The results (A−C) represent the means ± SE of three to eight separate experiments for each cell line. The data points from agonist-treated cells were fit to eq 1 by Prism 3. (D) Downregulation in pmol (24 h)$^{-1}$ (mg of protein)$^{-1}$ is plotted as a function of the initial β_1AR density, and each data point is from a separate experiment. The linear regression lines are shown, and the r^2 values are 0.9953 for ISO ($n = 15$) and 0.9739 for CPT-cAMP ($n = 10$).

Turnover of Human β_1AR. Downregulation may be due to increased receptor turnover or reduced synthesis (*28*). To have a reference, we determined the basal rates of human β_1AR synthesis (k_s) and turnover (k_{bsl}) in CHW cells using several different approaches. To directly measure receptor synthesis, we induced the cells with $ZnSO_4$. After a lag of ~2 h, receptor levels in both low and high β_1AR-expressing cells increased over the next 24 h with rates of net synthesis of 21 and 233 fmol h^{-1} (mg of protein)$^{-1}$ (Figure 4A). In addition, cells were exposed to an alkylating reagent that irreversibly inactivates β_1AR, and the rate of receptor recovery was measured [62 ± 7 fmol h^{-1} (mg of protein)$^{-1}$]. To directly determine the basal rate of β_1AR turnover, cells expressing low receptor levels were induced with increasing

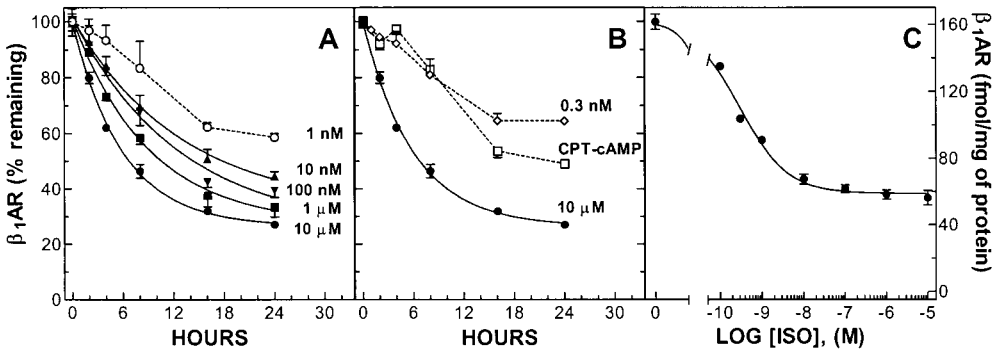

FIGURE 2: Effect of agonist concentration on downregulation of β_1AR in CHW-hβ_1 cells. Cells expressing low β_1AR levels were exposed for the indicated times (A, B) or 24 h (C) to increasing concentrations of ISO and assayed for total remaining binding sites. The curves through the time points were generated by Prism 3 using eq 1 (solid lines and symbols) except for 1 and 0.3 nM, which are point to point (dotted lines and open symbols; see text). For comparison, a time course of downregulation in cells exposed to 200 μM CPT-cAMP (□) is shown. The data represent the means ± SE of three to five experiments.

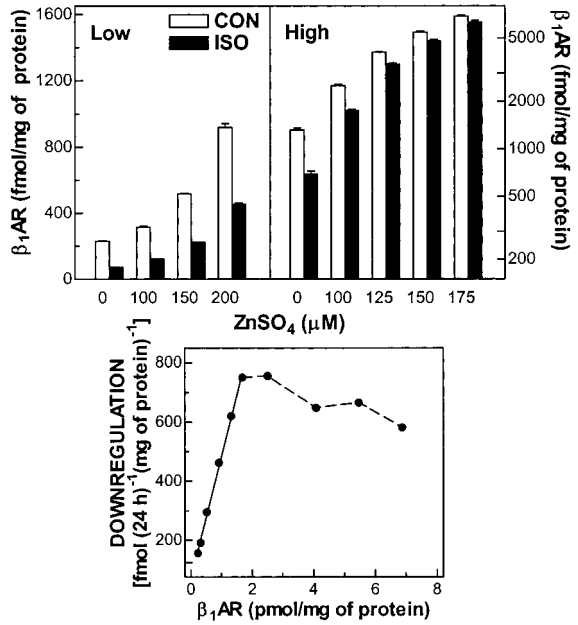

FIGURE 3: Effect of β_1AR induction by zinc on agonist-mediated downregulation in CHW-hβ_1 cells. (Top panel) Cells expressing low and high β_1AR levels were exposed to $ZnSO_4$ as indicated for 48 h, without (open bars) and with (solid bars) ISO for the last 24 h, and assayed for total binding. (Bottom panel) Downregulation plotted as a function of β_1AR density; the solid line represents the linear regression ($r^2 = 0.9981$). The results are from one of three similar experiments.

concentrations of $ZnSO_4$ for 28 h, then cultured in medium without added $ZnSO_4$ for different times, and assayed for β_1AR (Figure 4B). The cells lost their complement of induced receptors with first-order kinetics (eq 2a), and we used eq 2b to calculate values for k_s. Finally, we used eqs 3a and 3b to calculate values for both k_s and k_{bsl} from the downregulation data (Table 1). Values for k_s obtained by the four methods were fitted to the same linear relationship between rate of synthesis and β_1AR expression level (Figure 4C). The values for k_{bsl} and the corresponding $t_{1/2}$ values obtained from the downregulation data were similar to those obtained from the turnover of zinc-induced β_1AR (Table 1). The differences between the two sets of data were not significant, and using both sets of values, the mean half-life of the receptor was 18.9 ± 1.1 h. Thus, during agonist-mediated downregulation, β_1AR turnover (k_{dr}) increased between ~2- and 4-fold.

Role of Human β_1AR Internalization in Downregulation. As differences as to the role of endocytosis in β_2AR downregulation have been reported (*9*−*12*), we investigated whether internalization of β_1AR was required for its downregulation. Cells were exposed to agonist for short times and assayed for surface binding using the hydrophilic ligand [^{3}H]-CGP-12177. The percentage of surface receptors internalized was greater in low than in high β_1AR-expressing cells (Figure 5B). The loss of [^{3}H]CGP-12177 binding sites was due to internalization as [^{125}I]CYP binding was not reduced over the same period (Figure 5A). In contrast, no internalization occurred in cells exposed to CPT-cAMP for up to 1 h (Figure 5D). Even when high β_1AR-expressing cells were treated with CPT-cAMP for 24 h, surface binding and total binding were reduced the same to 65.3 ± 1.4% and 65.5 ± 3.8% of control ($n = 3$). In comparison, surface binding decreased 17.0 ± 2.7% more than total binding for agonist-treated cells, and this difference, representing internalized β_1AR, was similar to values after 20 min of agonist stimulation. As was found for downregulation, β_1AR internalization followed first-order kinetics (Table 1), and the amount internalized was directly proportional to the density of surface receptors up to ~ 3 pmol/mg of protein whereupon saturation was reached (Figure 6A). β_1AR internalization also was dependent on agonist concentration with an EC_{50} of 48 ± 12 nM (Figure 6B).

As both agonist-mediated internalization and downregulation of β_1AR followed first-order kinetics and were proportional to receptor density (Figures 1D and 6A), we compared the rates of the two processes by using Prism to extrapolate their initial rates. The respective rates [in fmol/mg of protein] were 14.0 per minute and 20.7 per hour for low β_1AR-expressing cells and 36.5 per minute and 56.75 per hour for cells with high β_1AR levels. Thus, β_1AR internalization occurred at an ~40-fold faster rate than β_1-AR downregulation. To further explore the role of internalization in downregulation, we used two well-established inhibitors of endocytosis of receptors including βAR. One was hypertonic sucrose which we had previously shown blocks β_1AR internalization in CHW cells (*20*), and the other was ConA (*30*−*32*). For these experiments, we used the low β_1AR-expressing cells (clone 1 in Table 1) because a substantial level (~50%) of agonist-mediated downregulation occurred by 6 h with only a small effect by CPT-cAMP (see

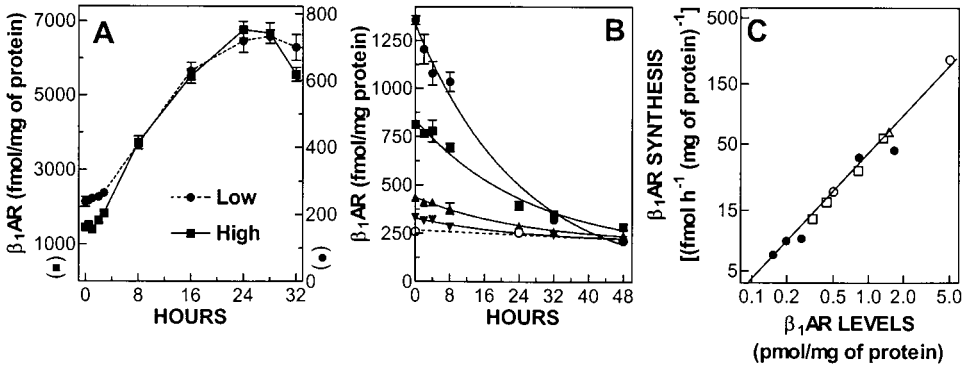

FIGURE 4: Synthesis and basal turnover of β_1AR in CHW-hβ_1 cells. (A) Cells expressing low (●) and high (■) β_1AR levels were exposed for the indicated times to 175 μM $ZnSO_4$ and assayed for specific ^{125}ICYP binding as described in Experimental Procedures. (B) Cells expressing low β_1AR levels were exposed for 28 h to 0 (○), 100 (▼), 125 (▲), 150 (■), and 175 (●) μM $ZnSO_4$ and then for the indicated times to regular medium and assayed for binding. Data are the means ± SD from one of two similar experiments and were fit to eq 2 by Prism 3. (C) The rate of β_1AR synthesis (k_s) was determined by different methods (see text) and plotted as a function of the relative receptor density from zinc induction (○), recovery after inactivation (△), turnover of zinc-induced receptors (□), and downregulation (●).

Table 1: Kinetic Parameters for Basal Turnover and Agonist-Mediated Internalization and Downregulation of β_1AR[a]

process	parameter	units	clone 1	clone 2	clone 3
downregulation	R_0	fmol/mg of protein	152 ± 1.9	267 ± 2.5	1658 ± 17
	R_f	fmol/mg of protein	40.0 ± 2.0	77.5 ± 5.5	719 ± 125
	k_{dr}	fraction/h	0.1687 ± 0.010	0.1157 ± 0.008	0.0621 ± 0.014
	$t_{1/2}$	h	4.11	5.99	11.2
	k_s	fmol h^{-1} (mg of protein)$^{-1}$	6.74 ± 0.68	8.97 ± 1.2	44.6 ± 17.8
	k_{bsl}	fraction/h	0.0444	0.0336	0.0269
	$t_{1/2}$	h	15.6	20.6	25.8
internalization	R_{en}	% of surface R	28.3 ± 0.91	32.1 ± 1.8	20.6 ± 1.0
	k_{en}	fraction/min	0.3613 ± 0.045	0.2054 ± 0.033	0.1262 ± 0.014
	$t_{1/2}$	min	1.92	3.38	5.49

			$ZnSO_4$ (μM)			
process	parameter	units	100	125	150	175
turnover of Zn-induced β_1AR	R_{ind}	fmol/mg of protein	332 ± 23	436 ± 16	812 ± 20	1336 ± 22
	k_{bsl}	fraction/h	0.0390	0.0398	0.0381	0.0417
	$t_{1/2}$	h	17.8	17.4	18.2	16.6
	k_s	fmol h^{-1} (mg of protein)$^{-1}$	12.9	17.4	30.9	55.6

[a] The kinetic parameters are derived from experiments shown in Figures 1, 4, and 5 by Prism 3 software as described in Experimental Procedures. The parameters for downregulation are as follows: R_0 and R_f, initial and final receptor levels; k_s, rate of receptor synthesis; k_{dr}, first-order rate constant for downregulation; k_{bsl}, rate constant for basal receptor turnover and their half-times where $t_{1/2}$ equals 0.693/k. Those for internalization are as follows: R_{en}, percent of surface receptors internalized; k_{en}, first-order rate constant for internalization with its $t_{1/2}$. Those for turnover of zinc-induced receptors are as follows: R_{ind}, initial level of induced receptors, rate constant and half-time for basal turnover, and rate of receptor synthesis.

Figure 1A). Furthermore, we observed that cells exposed to either 0.45 M sucrose or ConA began to round up after 8 h, indicating some toxicity. In this regard, we also tried treating the cells with monodansylcadaverine (*33*) and medium containing acetic acid at pH 5.0 (*11*), but both were very toxic. As shown in Figure 7A, both β_1AR internalization and downregulation were inhibited by ConA or hypertonic sucrose treatment. The latter was the more effective and completely blocked downregulation at all times tested (Figure 7B). Even after prolonged exposure to the inhibitors, the cells maintained the ability to produce cAMP upon agonist stimulation (Figure 7C). Taken together, the data indicate that internalization is required but not rate-limiting for agonist-mediated downregulation of β_1AR.

Downregulation of β_1AR mRNA Levels. To determine whether a reduction in β_1AR mRNA levels might contribute to β_1AR downregulation especially in response to CPT-cAMP, we used ribonuclease protection assays and a biotinylated riboprobe to quantify the levels in control and treated cells (Figure 8A). The 216 bp probe (lane 1) was totally digested by RNases in the presence of extracts of CHW-hβ_2 cells (lane 2) or tRNA (not shown), but when hybridized to sense strand (lanes 7−10), a protected fragment was detected in proportion to the amount of sense strand (Figure 8B). When the probe and a cyclophilin riboprobe (as the internal standard) were hybridized to CHW-hβ_1 cell extracts (lanes 3−6), protected fragments from both probes were recovered. Whereas the amount of the cyclophilin fragment was relatively constant, that of the β_1AR fragment varied, reflecting differences in receptor mRNA levels in the various cell samples. Cells expressing low β_1AR levels (lane 4) had 15−20% of the amount obtained from the high-expressing cells (lane 5) whereas zinc induction of the former increased the amount severalfold (lane 3) and agonist treatment of the latter cells decreased it (lane 6). In cells exposed to CPT-cAMP, β_1AR mRNA levels decreased with time, reached a new steady state of 43% of control by 6 h, and remained reduced for up to 24 h (Figure 8C). Similar results were observed in cells exposed to agonist, but the decreases were more variable and only reached 60% of

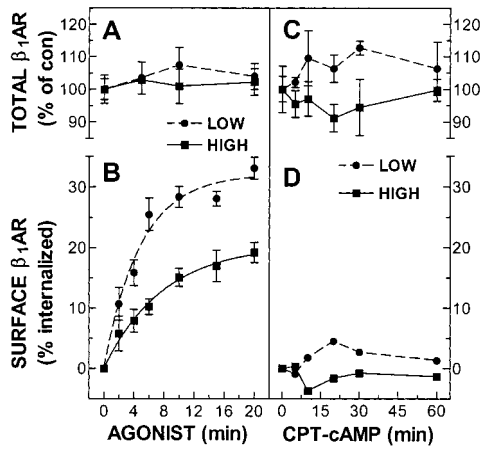

FIGURE 5: Effect of agonist and CPT-cAMP on β_1AR internalization in CHW-hβ_1 cells. Cells expressing low (●) and high (■) β_1AR levels were exposed to ISO (A, B) or CPT-cAMP (C, D) for the indicated times, and either intact cells were assayed for surface receptors using [^{3}H]CGP-12177 (B, D) or lysates were assayed for total receptors using ^{125}ICYP (A, C) as described in Experimental Procedures. The results represent the means ± SE of three to five separate experiments.

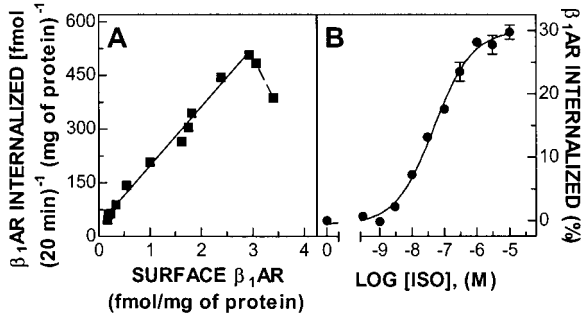

FIGURE 6: Effect of receptor density and agonist concentration on internalization of β_1AR in CHW cells. (A) Cells expressing different β_1AR levels were incubated in the absence and presence of ISO for 20 min and assayed for [^{3}H]CGP-12177 binding as described in Experimental Procedures. Internalization in fmol (20 min)$^{-1}$ (mg of protein)$^{-1}$ is plotted as a function of the initial receptor density. The linear regression line has an r^2 value of 0.9892. (B) Cells expressing a low density of β_1AR were exposed to the indicated concentrations of ISO for 20 min and assayed for surface receptors. Data are the means ± SD from one of two similar experiments.

control (Figure 8D). This may be a consequence of agonist-mediated desensitization, internalization, and even downregulation of β_1AR that reduces agonist stimulation of adenylyl cyclase and generation of cAMP. Thus, CPT-cAMP was more effective than agonist in reducing β_1AR mRNA levels which is the reverse of their effects on downregulation of receptor binding activity. The most likely mechanism for downregulation of β_1AR mRNA in cells expressing the β_1-AR cDNA under the control of a nonendogenous promoter is mRNA destabilization (*13*−*15*, *34*). We tested this possibility by blocking transcription in control and treated cells with actinomycin D and measured β_1AR mRNA levels at increasing times (Figure 8E). Agonist treatment significantly reduced the $t_{1/2}$ of the β_1AR transcripts from 7.83 ± 0.4 to 4.54 ± 0.2 h ($p < 0.0001$, $n = 6$).

Downregulation of β_1AR by Western Blotting. To follow the fate of the receptor protein, we used immunoblotting with antibodies to either unmodified or carboxyamidated peptides corresponding to a 20 amino acid C-terminal sequence of the human β_1AR (Ala^{456}-Ser^{475}) as well as a commercial antibody to the same unmodified peptide. The specificity of the antibodies can be seen in Figure 9A. No immunoreactive products were detected in high-expressing CHW-hβ_1 cells blotted with preimmune serum (lane 1) whereas two proteins with apparent molecular masses of 64 and 54 kDa[2] were detected with antiserum from the same rabbit immunized with the amidated peptide (lane 3). As additional negative controls, we blotted lysates from CHW cells either untransfected (lane 4) or expressing high β_2AR levels (lane 2). Staining of the two proteins was less intense in lysates from low receptor-expressing cells (lane 6) and more intense from zinc-induced cells (lanes 5 and 7). There was some indication of receptor dimerization in the induced, high β_1AR-expressing cells (lane 5). The commercial antibody as well as antiserum PF-13 (data not shown), both to the unmodified peptide, detected the same two proteins in CHW-hβ_1 cell lysates but with less sensitivity (compare lane 9 with lane 3) and did not react with CHW cell proteins (lane 8). To further characterize the two immunoreactive proteins, we subjected CHW-hβ_1 cells to surface biotinylation with sulfo-NHS-LC-biotin or biotin-LC-hydrazide after $NaIO_4$ oxidation to label amino or oligosaccharide groups on β_1AR followed by detergent solubilization and immunoprecipitation with the anti-β_1AR antiserum. With either labeling technique, both proteins were detected on blots by horseradish peroxidase-conjugated streptavidin (Figure 9B), indicating that both forms of β_1AR are expressed on the cell surface, glycosylated, and available for ligand binding. We next tested the antiserum raised against the amidated peptide for sensitivity and proportionality (Figure 9C). As little as 2.5 fmol of β_1AR binding activity could be detected, and the chemiluminescent signal increased linearly over a 10-fold range of β_1AR. In addition, we found that the different cells produced similar immunoreactive responses per femtomole of binding activity; the relative responses were 100, low; 103, low induced; 110, high; and 95.5, high induced.

When we did Western blotting on lysates from CHW-hβ_1 cells exposed to either agonist or CPT-cAMP for 24 h, we observed a decrease in β_1AR immunoreactivity (Figure 10A). With the latter treatment, loss of immunoreactivity in either the low or high β_1AR-expressing cells was very modest (~20%) and less than the loss of binding activity. With agonist treatment, the low receptor-expressing cells exhibited the largest decrease in β_1AR immunoreactivity that was somewhat less than the downregulation in binding. In the high β_1AR-expressing cells, the decrease in both parameters was very similar. Despite the extensive loss of β_1AR protein, no immunoreactive degradation products were detected. When we determined the time course of agonist-mediated downregulation of β_1AR protein (Figure 10B−D), it was apparent that loss of receptor binding activity preceded that of receptor protein. The differences were more pronounced for the cells expressing low β_1AR levels, which may be a result of the more rapid kinetics of downregulation in the low receptor-expressing cells compared to those expressing high levels of β_1AR.

[2] Two proteins of similar molecular masses were observed in Chinese hamster ovary and HEK 293 cells stably and transiently expressing the human β_1AR, respectively (*35*, *36*).

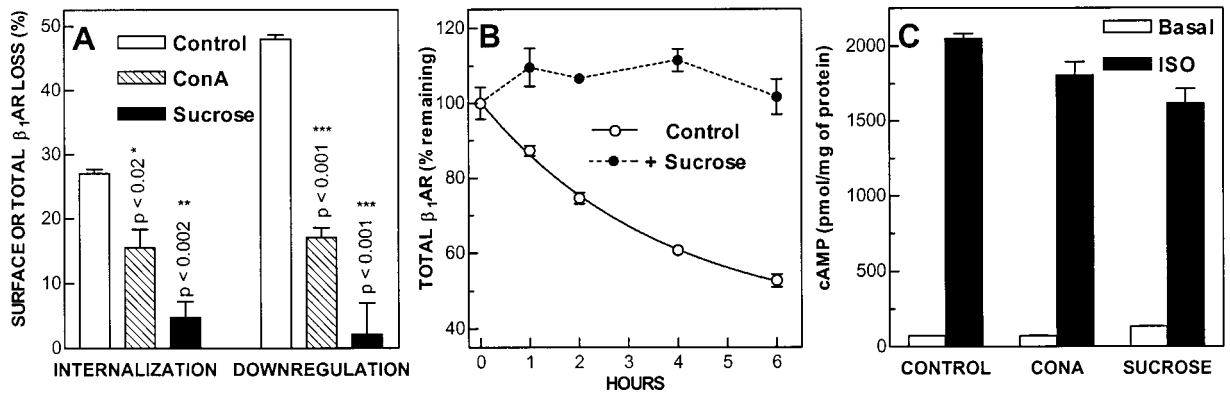

FIGURE 7: Effect of inhibitors of internalization on downregulation and functional activity of β_1AR in CHW cells. (A) Cells were exposed to no inhibitor, ConA, or hypertonic sucrose for 30 min, then incubated with and without 10 μM ISO, and assayed for internalization after 30 min or for downregulation after 6 h as described in Experimental Procedures. Data are the means ± SE of three separate experiments. (B) Control (○) and sucrose-treated (●) cells were exposed to 10 μM ISO for the indicated times and assayed for total binding. Data are the means ± SD from one of two similar experiments. (C) Same as in panel A except after 6.5 h the cells were assayed for basal and agonist-stimulated cAMP. Data are the means ± SE of two separate experiments. In a third using clone 2 (Table 1), neither inhibitor reduced the cAMP response.

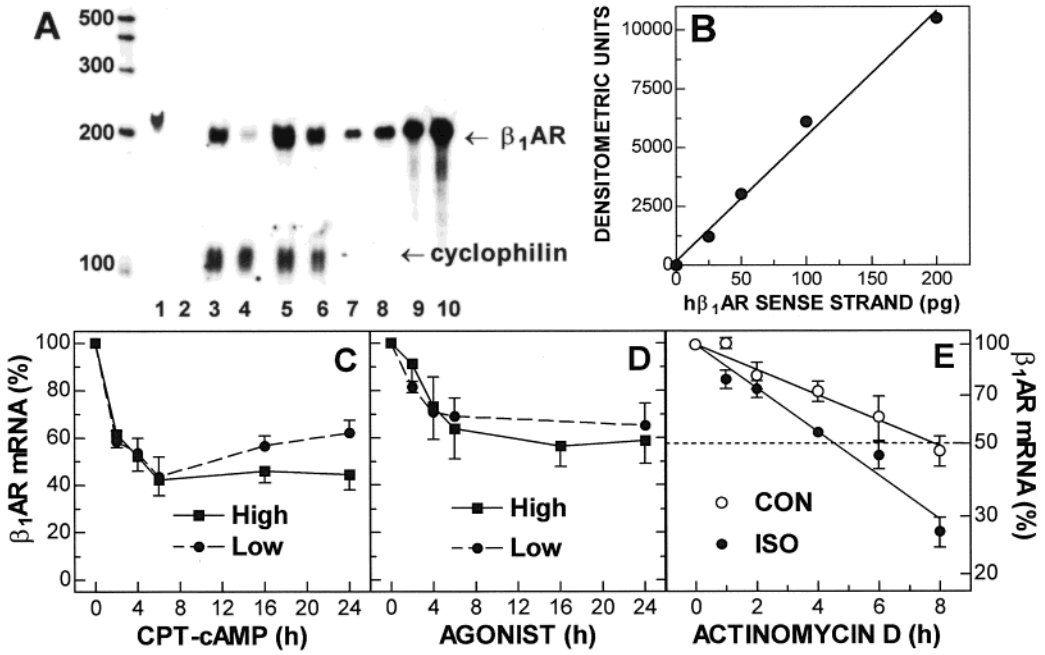

FIGURE 8: Agonist- and cAMP-induced reduction in β_1AR mRNA levels and stability in CHW-hβ_1 cells. (A) A representative ribonuclease protection assay using biotinylated β_1AR (all lanes) and cyclophilin (lanes 3−6) riboprobes as described in Experimental Procedures is shown. The probes were hybridized as indicated, digested with RNases (except lane 1), resolved by gel electrophoresis, transferred to nylon, and detected by alkaline phosphatase−streptavidin chemiluminescence. The mRNA-protected fragments are 195 and 103 bp. Lanes: 1, free β_1AR riboprobe; 2−6, extracts of cells expressing β_2AR; low β_1AR levels, zinc-induced and control; high β_1AR levels, control and treated with ISO for 24 h; 7−10, 25−200 pg sense strand. (B) The densitometric quantification of the sense strand-protected fragment. Cells expressing low (●) and high (■) β_1AR levels were exposed to CPT-cAMP (C) or ISO (D) for the indicated times and assayed for β_1AR mRNA levels. (E) Cells expressing high β_1AR levels were incubated for 24 h in the absence (○) and presence (●) of ISO, then exposed to actinomycin D for the indicated times, and assayed for β_1AR mRNA levels. The results represent the means ± SE of three to six separate experiments.

In a study on downregulation of human β_2AR, a similar delay is observed and attributed to a pool of immunoreactive but nonbinding receptors found in the light membrane fraction (*11*). To determine whether a similar pool of β_1AR existed, we separated the light endosomal membranes from the heavy plasma membranes by centrifugation. When we compared β_1AR binding activity with immunoreactivity for the two fractions along with the cell lysate and postnuclear supernatant, we found that the heavy membranes had more immunoreactivity than the cell lysate for equal amounts of binding activity (Table 2). The values for the postnuclear supernatant and the light membrane fractions were similar to that of the cell lysate.

Effect of Protease Inhibitors on hβ1AR Downregulation. Finally, we explored whether protease inhibitors could impede agonist-mediated downregulation of β_1AR, as there have been conflicting reports about their effects on β_2AR downregulation. In HEK 293 cells expressing human HA-β_2AR, leupeptin but not pepstatin was found to inhibit agonist-mediated downregulation of binding activity (*10*). In contrast, leupeptin was found to have no effect on downregulation of the same receptor expressed in L cells

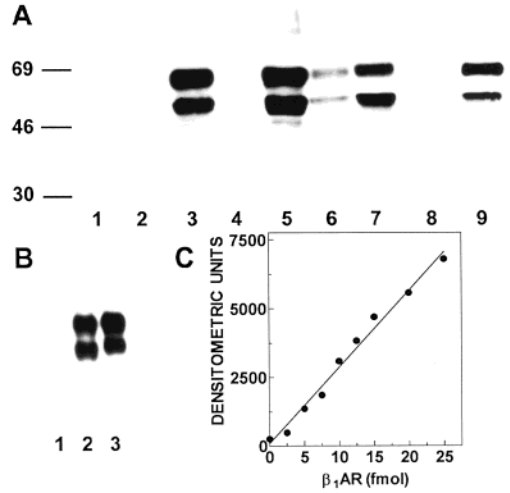

FIGURE 9: Western analysis of human β_1AR with anti-C-terminal peptide antibodies. (A) Portions (25 μg of protein except 8.3 μg for lane 5) of lysates from CHW cells that were untransfected (lanes 4 and 8) or expressing low β_1AR (lane 6) and zinc-induced (lane 7), high β_1AR (lanes 1, 3, and 9) and zinc-induced (lane 5), and high β_2AR (lane 2) were subjected to SDS−PAGE and immunoblot analysis with preimmune serum (lane 1), antiserum PF-11 to the carboxyamidated peptide (lanes 2−7), and antibody A20 to the native peptide (lanes 8−9) and visualized by chemiluminescence as described in Experimental Procedures. (B) Soluble extracts (4 μg of protein, 21−27 fmol) from zinc-induced, high β_1AR-expressing cells that were untreated (lane 1) and surface biotinylated with sulfo-NHS-LC-biotin (lane 2) or biotin-LC-hydrazide (lane 3) were immunoprecipitated with antiserum PF-11, and the precipitates were subjected to blotting with HRP-conjugated streptavidin and visualizing by chemiluminescence. (C) Increasing amounts of lysate from high β_1AR-expressing cells were subjected to immunoblotting with antiserum PF11 and visualized by chemiluminescence. The images were quantified by densitometric analysis and the values plotted as a function of fmol of β_1AR binding activity.

Table 2: Subcellular Distribution of β_1AR Binding Activity and Immunoreactivity in CHW-hβ_1 Cells[a]

fraction	binding activity (%)	immunoreactivity (*x*-fold)
lysate	100.0	1.00
postnuclear supernatant	99.2 ± 5.7	0.96 ± 0.18
heavy membranes	48.7 ± 2.7	2.74 ± 0.27
light membranes	13.2 ± 2.9[b]	1.28 ± 0.15

[a] Lysates of high β_1AR-expressing cells were centrifuged at 450*g* for 10 min, and the resulting postnuclear supernatants were separated into light and heavy membrane fractions as described in Experimental Procedures. Portions (20 fmol of binding activity) were analyzed by immunoblotting and quantified by densitometry. The results represent the means ± SE of four separate experiments. [b] Based on binding of [^{3}H]CGP-12177 to intact cells and binding of ^{125}ICYP to lysates of the same cells; 85% of β_1AR was on the cell surface.

(*11*). When we exposed CHW-hβ_1 cells to leupeptin as well as E-64 (data not shown), another cysteine protease inhibitor, we did not observe any inhibition of downregulation (Table 3). The effect was not subtype-specific as similar results were obtained with CHW-hβ_2 cells. We were able to reproduce the observations of Moore et al. (*10*) when we used HEK 293 cells expressing either human wild-type or HA-β_2AR. For reasons yet to be elucidated, when we expressed human β_1AR in HEK 293 cells, it failed to undergo agonist-mediated downregulation. We did find that endogenous β_1AR and β_2AR in human SK-N-MC and A431 cells, respectively, underwent agonist-mediated downregulation, and there was

Table 3: Effect of Leupeptin on Downregulation of βAR Binding Activity in Different Cell Lines[a]

cell line	βAR binding activity (fmol/mg of protein)			
	−Leu/−ISO	−Leu/+ISO	+Leu/−ISO	+Leu/+ISO
CHW-hβ_1 (low)	225 ± 5	98 ± 5	223 ± 6	109 ± 2
CHW-hβ_2 (low)	217 ± 5	45 ± 1	203 ± 16	40 ± 1
CHW-hβ_1 (high)	1654 ± 40	906 ± 24	1646 ± 15	962 ± 18
CHW-hβ_2 (high)	1489 ± 16	840 ± 11	1367 ± 42	859 ± 20
HEK 293-hβ_2HA	1287 ± 36	655 ± 17	1295 ± 4	1004 ± 9
HEK 293-hβ_2	485 ± 19	182 ± 28	524 ± 26	400 ± 41

[a] Cells were exposed to 100 μM leupeptin for 1 h and to 10 μM ISO for 24 h as indicated. Then lysates were prepared and assayed for ^{125}ICYP binding as described in Experimental Procedures. The results are from one of two to three similar experiments. Cell lines used were CHW cells stably expressing low and high levels of either β_1AR or β_2AR and HEK 293 cells either stably expressing HA-β_2AR or transiently expressing wild-type β_2AR.

no inhibition by leupeptin (data not shown). Furthermore, when we exposed CHW-hβ_1 or HEK 293-hβ_2 cells to calpain inhibitor I for up to 24 h, we did not observe any increase in receptor binding activity or any prevention of agonist-mediated downregulation. This is in contrast to the 15-fold increase in β_2AR observed in L cells by Jockers et al. (*11*). Thus, the effects of protease inhibitors may be cell-specific.

Comparison of Human β_1AR and β_2AR Downregulation. It also is evident from Table 3 that when both β-subtypes are expressed in the same cell line at similar low levels, β_2-AR undergoes more downregulation than β_1AR as reported previously (*18*, *20*). Downregulation of β_2AR also was more rapid at both low and high expression levels with $t_{1/2}$ values of 2.57 and 7.25 h. Despite this difference, downregulation of the two subtypes in CHW cells appears to involve similar mechanisms. Both downregulate in response to cAMP after a delay of several hours, without internalization, and with a corresponding reduction in receptor mRNA levels (present study and ref *37*). We also found that hypertonic sucrose, which blocks agonist-mediated internalization of β_2AR (*20*), inhibited its downregulation. After 4 h with agonist, β_2AR binding was 105 ± 10% of control compared to 55 ± 4.3% in cells not exposed to sucrose ($n = 3$). Finally, we observed a decrease in β_2AR immunoreactivity during downregulation (with an antibody raised against the C-terminus of β_2AR). For cells expressing high levels of β_2AR, there was a 51 ± 5% decrease after 24 h of agonist treatment ($n = 3$).

DISCUSSION

Even though the phenomenon of downregulation of βAR has been investigated for several decades, little is known about the underlying mechanism(s) and the site(s) involved or the fate of the receptor protein. Regarding the latter, two earlier studies gave conflicting results. One found that agonist treatment of hamster DDT$_1$ MF-2 cells causes a loss of endogenous β_2AR based on immunofluorescence with anti-β_2AR antibodies (*5*). The other reported that, in agonist-treated human A431 cells, endogenous β_2AR binding activity decreases with no change in immunoreactivity by Western blotting (*6*). More recently, several studies have identified some aspects of the intracellular trafficking of β_2AR in agonist-stimulated cells (*7*−*10*). The receptors undergo endocytosis through the clathrin-mediated pathway and

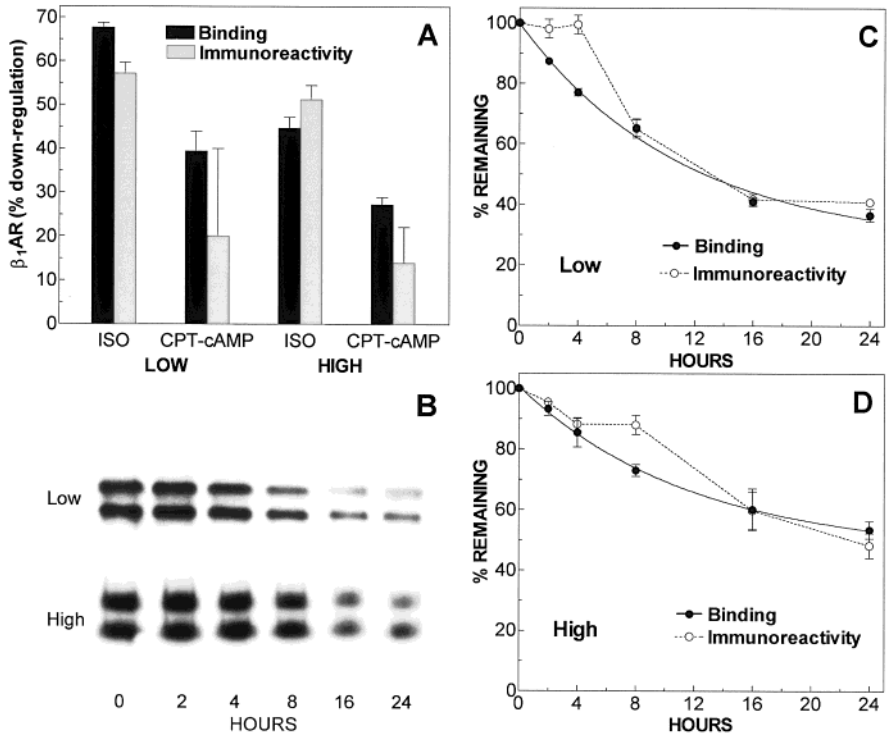

FIGURE 10: Comparison of downregulation of β_1AR binding activity and immunoreactivity in CHW-hβ_1 cells. (A) Cells expressing low and high β_1AR levels were exposed to no addition, ISO, or CPT-cAMP for 24 h and assayed for binding and for immunoreactive proteins as described in Experimental Procedures. Densitometric quantification and binding activity are presented as means ± SE of four to seven separate experiments. (B−D) Same as in panel A except cells were exposed to ISO for the indicated times. Representative blots are shown in panel B, and densitometric quantification and binding activity as means ± SE of four to five separate experiments are presented in panels C and D.

eventually end up in lysosomes. None of these studies addressed the fate of the receptor protein. Furthermore, the requirement for endocytosis may not be universal as downregulation of β_2AR in murine L and A431 cells occurs even when endocytosis is blocked (*11*). In this latter study, degradation of HA-β_2AR expressed in L cells was detected by immunoblotting crude membrane fractions with anti-HA antibody.

In the present study, we investigated the downregulation of the human β_1AR, about which even less is known. We found downregulation of β_1AR to be very complex with reductions in receptor binding activity, mRNA levels, and immunoreactive protein. In addition, downregulation occurred in response to both agonist and a permeable cAMP analogue. In cells exposed to the latter, there was less downregulation as well as a delay of several hours before a reduction in β_1AR levels was observed. A similar pattern has been observed for human β_2AR expressed in CHW cells (*37*). These authors also found that β_2AR mRNA is downregulated, presumably due to increased destabilization by an mRNA binding protein(s) induced by cAMP (*13*−*15*, *34*). In CHW-hβ_1 cells, we showed that the decrease in β_1AR mRNA levels was due to reduced stability as indicated by the more rapid turnover. Taken together, our results indicate that agonist-mediated downregulation of β_1AR in CHW cells includes both agonist- and cAMP-specific components. This is very apparent from the effect of agonist concentration on the time course of downregulation. Whereas at concentrations above 1 nM loss of binding followed a first-order decay curve, at or below 1 nM downregulation occurred after a lag of several hours and appeared similar to that mediated by cAMP. Most likely, the cAMP-specific component of downregulation is due mainly to the decrease in β_1AR mRNA levels followed by a reduction in receptor synthesis. Loss of β_1AR then becomes dependent on basal turnover that is relatively slow with a $t_{1/2}$ of 20 h. To test this, we modified eq 2a to include both a delay of 4 h and a reduction in k_s to approximate the changes in β_1AR mRNA levels (Figure 8). For k_{bsl}, we used the values from Table 1. The curves generated by Prism 3 fit the data points for downregulation mediated by CPT-cAMP as well as 0.3 and 1 nM isoproterenol fairly well. Although we cannot eliminate the possibility that phosphorylation of β_1AR by PKA contributes to its downregulation, we believe that it is unlikely. Phosphorylation is a very early event as evidenced by the PKA-mediated desensitization of β_1AR (*20*) whereas downregulation is not observed for several hours. In addition, cAMP did not induce β_1AR internalization over the 24 h period that cAMP-mediated downregulation was observed.

When we examined the kinetics of agonist-mediated downregulation of β_1AR, we found effects not only of agonist concentration but also of receptor density. As expected, the rate of downregulation increased between 10 nM and 10 μM isoproterenol ($t_{1/2}$ of 9 vs 4.1 h) whereas the opposite effect was observed when downregulation was compared between cells expressing low and high receptor levels ($t_{1/2}$ of 4.1 vs 10.2 h). When the extent of downregulation was expressed as amount of receptor per 24 h, however, the loss of binding was proportional to the initial levels of β_1AR up to ~2 pmol/mg of protein after which downregulation reached a plateau. Thus, downregulation of β_1AR in CHW cells appears to be a saturable process, but only at a high level of expression.

Although we found that low and high receptor-expressing cells exhibited corresponding differences in agonist-mediated

internalization of their surface β_1AR, the receptors were internalized at rates ~40-fold faster than the rates of downregulation. As we found for the latter process, the number of receptors internalized per 20 min was proportional to the initial surface β_1AR density and reached saturation but a higher level (~3 pmol/mg of protein). Thus, β_1AR internalization was not rate-limiting for downregulation. When we blocked agonist-mediated internalization, however, we prevented the downregulation, indicating that the former is required for the latter. Our results are in contrast to those of Jockers et al. (*11*), who found that blocking internalization does not inhibit the downregulation of human β_2AR in either L or A431 cells. As we also found that internalization was necessary for human β_2AR downregulation in CHW cells, the differing results may be due to use of different cell lines. In addition, we observed that internalization did not occur in cells exposed to CPT-cAMP and thus appears to be agonist-specific. In this regard, the EC_{50} value for agonist-mediated internalization was more than 100-fold higher than for downregulation and more closely related to agonist occupancy of β_1AR. Thus, low agonist concentrations sufficient to raise cAMP levels were able to induce downregulation but not internalization. Finally, if β_1AR is being internalized faster than it is being downregulated yet at any given time only 20−30% of the total receptors are not on the cell surface, then most of the internalized β_1AR probably is being recycled back to the plasma membrane even in the continual presence of agonist. This is consistent with studies on the human β_2AR (*38*).

Using antibodies specific for human β_1AR and immunoblotting of cell lysates, we observed a time-dependent loss of receptor protein in agonist-treated cells. In this regard, our goal was to determine the fate of wild-type receptor protein and to analyze all of the receptors in the cell. Although we cannot distinguish between total receptor degradation and partial proteolytic cleavage of the C-terminal antigenic determinant, we did not find any smaller immunoreactive fragments on the blots. We did observe that the disappearance of β_1AR immunoreactivity was delayed relative to the loss of binding activity. One explanation could be the pool of inactive receptors that we detected when we compared light and heavy membrane fractions by immunoblotting. These may represent newly synthesized receptors that have not yet assumed an active conformation or mature receptors that are in the process of being turned over. Regardless of its source, this pool initially may be masking the decrease in active β_1AR protein detected by immunoblotting. A similar phenomenon was described in L cells expressing HA-β_2AR except the pool of inactive receptors was detected in the light membrane fraction (*11*). Again, this may be due to the use of different cells.

While protease inhibitors potentially may be useful in delineating the site(s) of downregulation, differing results have been reported on their effects on β_2AR downregulation. Leupeptin and E-64, two inhibitors of cysteine proteases, as well as calpain inhibitor I, which is also a proteasome inhibitor, were found to be ineffective in preventing downregulation of HA-β_2AR expressed in L cells (*11*). In contrast, leupeptin inhibits the loss of binding activity of HA-β_2AR expressed in HEK 293 cells (*10*). In the presence of leupeptin, the receptors are observed to accumulate in lysosomes when detected with anti-HA antibody and visualized by immunofluorescence. We confirmed that leupeptin (and E-64 but not calpain inhibitor I) inhibited downregulation in HEK 293 cells expressing HA-tagged as well as wild-type β_2AR. The ability of leupeptin to block the loss of binding activity may be cell-specific as we found no effect in either CHW-hβ_1 and CHW-hβ_2 cells or human SK-N-MC and A431 cells expressing endogenous β_1AR and β_2-AR, respectively.

Given the complexity of agonist-mediated downregulation of the human β_1AR expressed in CHW cells, there does not appear to be a simple model that encompasses all of the various aspects. At a minimum, the model includes both agonist- and cAMP-specific components. Upon further scrutiny of the data, it is apparent that the agonist-specific component of β_1AR downregulation not only requires internalization of the receptor but also predominates at high agonist concentrations, low receptor levels, and the earlier times of exposure. In contrast, the cAMP-specific component does not require internalization and is more effective at low agonist concentrations, high receptor levels, and the later times of exposure. In this regard, our model for downregulation of the human β_1AR expressed in CHW cells shares several similarities with the kinetic model for agonist-mediated downregulation of endogenous β_2AR in human BEAS-2B epithelial cells developed by Williams et al. (*12*). Their scheme also has two components: a high-affinity (EC_{50} = 0.5 nM), low-capacity one that is independent of internalization and a low-affinity, high-capacity one that is closely linked to internalization. They further suggest that the high-affinity component involves cAMP and PKA, which agrees with our model. One apparent difference is that, in our model, both components appear to have similar capacities (see Figure 1D).

It is important to point out that this two-component model for downregulation of βARs may be highly cell-specific and only occur in cells that undergo cAMP-mediated downregulation of receptors (and receptor mRNA) such as CHW (present study and ref *37*), rat C6 glioma (*16*, *39*), hamster DDT_1 MF-2 (*13*), and possibly human BEAS-2B (*12*) cells. It does not appear to operate in murine L (*11*), HEK 293 (unpublished observations and ref *10*), A431 (unpublished observations and ref *11*), and SK-N-MC (*25*, *40*) cells. In some of these cells, only the agonist-specific pathway for downregulation may be functioning. In others, downregulation may be through a total distinct pathway (*11*). Thus, one of the major challenges will be to resolve the cell-specific aspects of βAR downregulation. In this regard, we are exploring the basis for the downregulation of β_2AR but not β_1AR in HEK 293 cells in comparison to CHW cells in which both subtypes undergo downregulation. While GPCRs have specific cytoplasmic sequences (sorting signals) that mediate their targeting to either recycling or degradative pathways (*7*), regulation of intracellular trafficking also involves specific sorting proteins that recognize these signals. Variations in their levels of expression may contribute to cell-specific effects on downregulation.

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