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Use of a Density Shift Method to Assess *Beta*-Adrenergic Receptor Synthesis during Recovery from Catecholamine-Induced Down-Regulation in Human Astrocytoma Cells

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SUMMARY

Exposure of postconfluent 1321N1 human astrocytoma cells to 1.0 μM isoproterenol for 12–24 hr results in a 90% loss of *beta*-adrenergic receptors. Upon removal of agonist, recovery of *beta*-receptors to control levels occurs within 72 hr. The recovery of receptors is completely blocked by cycloheximide [R. C. Doss, J. P. Perkins, and T. K. Harden, *J. Biol. Chem.* **256**:12281–12286 (1981)]. In contrast cycloheximide does not block recovery of *beta*-receptors after down-regulation in preconfluent cultures. To determine unambiguously if *beta*-receptor synthesis accounts for the recovery of receptors after down-regulation, post confluent cultures were incubated with isoproterenol and then transferred to agonist-free medium containing either normal or "heavy" (^{2}H, ^{13}C, ^{15}N) amino acids. The rate and extent of *beta*-receptor recovery were similar in both normal and heavy amino acid-containing medium. When *beta*-receptors that had recovered in the heavy amino acid-containing medium were labeled with ^{125}I-cyanopindolol, solubilized in Lubrol PX, and subjected to centrifugation on a 5–15% sucrose density gradient, they exhibited an increased mass compared to *beta*-receptors that recovered in the presence of normal amino acids. These results confirm that the density shift method is a useful approach for the study of *beta*-receptor synthesis and that new receptor synthesis occurs during recovery of *beta*-receptors from catecholamine-induced down-regulation in postconfluent cultures.

INTRODUCTION

Exposure of target cells to *beta*-adrenergic receptor agonists induces a series of reactions that results in a major loss of catecholamine-stimulated adenylate cyclase activity (1, 2). In 1321N1 human astrocytoma cells, short term (in minutes) catecholamine-induced desensitization does not involve receptor loss per se (3). Instead, the plasma membrane receptors rapidly are converted to another membrane form that apparently represents internalized *beta*-receptors (4, 5). Reversal from short term desensitization (3) occurs rapidly ($t_{1/2} \sim 6$ min). In contrast, loss of responsiveness during long term exposure to *beta*-receptor agonists in this (6–8) and a number of other cells (9, 10) involves a loss of measurable receptors; in 1321N1 cells (7), recovery from this state upon removal of agonist from the medium is relatively slow ($t_{1/2}$ = 12 hr). The role of protein degradation and synthesis in the process of *beta*-receptor loss and recovery during long term desensitization has not been determined unambiguously. Nonetheless, it has been demonstrated in several cell types that agonist-induced loss of *beta*-adrenergic receptors and/or catecholamine-stimulated adenylate cyclase activity (9–11) and recovery from long term desensitization (7, 11) can be blocked by protein synthesis inhibitors.

Our studies of *beta*-receptors following long term exposure of 1321N1 astrocytoma cells to catecholamines indicate that the properties of the recovery process are dependent upon the density of the cultures. In preconfluent cultures, cycloheximide (7) and tunicamycin[3] at concentrations that inhibit protein synthesis and glycosylation by greater than 90%, and inhibit *beta*-receptor accumulation in dividing cultures, fail to prevent recovery of "lost" receptors. Thus, long term exposure of preconfluent 1321N1 cell cultures to catecholamines apparently results in the conversion of *beta*-receptors to a form that is no longer detectable by conventional radioligand-binding assays but that, nonetheless, retains its primary amino acid structure. The situation in post-

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confluent cultures is much different. That is, recovery of lost receptors is blocked by cycloheximide (7) but not by tunicamycin.[3] One interpretation of these results is that agonist-induced loss of *beta*-receptors in postconfluent 1321N1 cell cultures involves receptor degradation, and as a consequence, recovery from this state requires new receptor synthesis. The lack of effect of tunicamycin on receptor recovery suggests that either *beta*-receptors are synthesized as aglycoproteins in postconfluent cultures or that receptors synthesized as aglycoproteins in the presence of tunicamycin are distributed to the plasma membrane at the same rate and with the same binding and activity properties as the glycoreceptors synthesized in the absence of tunicamycin.

Experiments that involve the use of protein synthesis or glycoprotein synthesis inhibitors to study the regulation of the expression of a specific protein have considerable drawbacks. Most importantly, it is difficult to rule out the possibility that cycloheximide or tunicamycin modifies the expression of some unknown protein(s) that is important in the regulation of the specific protein in question. In recent years technology involving the use of heavy (^{2}H-, ^{13}C-, ^{15}N-labeled) amino acids has been applied by Fambrough and coworkers (12, 13) and by Lane and coworkers (14, 15) to the study of acetylcholine and insulin receptors. We have now applied this approach to confirm the role of protein synthesis in the recovery of *beta*-receptors following long term exposure of 1321N1 astrocytoma cells to catecholamines.

MATERIALS AND METHODS

Materials. Heavy isotope-labeled algal amino acid extract (80 atom % ^{13}C; 90 atom % ^{15}N; and 98 atom % ^{2}H) was purchased from Merck, Sharp and Dohme (Montreal, Canada). Normal isotope amino acids were from Calbiochem (San Diego, CA). Tissue culture dishes were from Falcon and ultrapure sucrose was from Schwarz-Mann. Na^{125}I was obtained from Amersham. (−)-Cyanopindolol was a generous gift from Gunther Engel, Sandoz (Basel). All other materials were obtained from sources previously cited (5, 7).

Cell culture. Human astrocytoma cells (1321N1) were grown in the absence of antibiotics on 150-mm plastic culture dishes (Falcon) in 20 ml of DMEM[4] supplemented with 5% fetal calf serum. Cells were subcultured at a density of 8,000–12,000 cells/cm^2 by detaching confluent monolayers with a solution of 0.025% trypsin in 100 mM NaCl, 10 mM sodium citrate buffer (pH 7.8). The cultures were maintained in a 37° humidified incubator in an atmosphere of 92% air and 8% CO_2. Three days after subculture, the growth medium was aspirated and replenished with fresh DMEM containing 5% serum.

Catecholamine-induced down-regulation. Postconfluent cultures were incubated on the 5th day after subculture with either (final concentrations) 1 mM ascorbate (control) or 1 mM ascorbate plus 1 μM (−)-isoproterenol (desensitized). Incubations were at 37° for 24–30 hr.

Recovery. The isoproterenol and ascorbate were removed by washing the cultures three times with 10 ml of HEPES (20 mM)-buffered Eagle's medium and once with phosphate-buffered saline (140 mM NaCl, 2.69 mM KCl, 1.47 mM KH_2PO_4, and 15.3 mM Na_2HPO_4). Some cultures were then harvested to document the extent of agonist-induced receptor loss. Other cultures were incubated an additional 60 hr in agonist-free DMEM with 5% serum containing either "heavy" isotope amino acids (95% enriched in ^{15}N, ^{13}C, and ^{2}H) or "normal" amino acids (^{14}N, ^{12}C, and ^{1}H).

[4] The abbreviations used are: DMEM, Dulbecco's modification of Eagle's medium; ^{125}I-CYP, ^{125}I-cyanopindolol; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

Preparation of normal and heavy isotope medium. Both normal and heavy isotope amino acids were dissolved in water, adjusted to pH 7.4, and filtered through an Amicon YM-2 ultrafiltration membrane. The final millimolar concentrations of these amino acids in the medium were: alanine, 1.27; arginine, 0.35; aspartic acid, 0.91; glutamic acid, 0.85; glycine, 0.82; histidine, 0.07; isoleucine, 0.51; leucine, 1.00; lysine, 0.28; methionine, 0.19; phenylalanine, 0.32; proline, 0.37; serine, 0.56; threonine, 0.63; tyrosine, 0.32; and valine, 0.64. Normal and heavy amino acid medium were both supplemented with millimolar concentrations of four essential amino acids: cysteine, 0.34; glutamine, 3.99; histidine, 0.05; and tryptophan, 0.08. Other medium components included (final millimolar concentrations): $CaCl_2$ (anhydrous), 1.80; $Fe(NO_3)_3 \cdot 9H_2O$, 0.0002; KCl, 5.36; $MgSO_4 \cdot 7H_2O$, 0.8; NaCl, 104; $NaH_2PO_4 \cdot H_2O$, 0.91; $NaHCO_3$, 44; glucose, 5.55; phenol red, 1.0; pyruvic acid, 1.0; and minimal essential medium vitamin solution (100× concentrate; GIBCO), 0.04 (ml/ml). The medium was sterilized by filtration through a Nalgene type S (0.2 μm) membrane prior to use.

Preparation of lysates. Cells were swollen hypotonically by washing once with 10 ml of ice-cold lysis buffer (1 mM Tris, 2 mM EDTA, pH 7.5) and incubating for 20 min in an additional 10 ml of buffer. The cells were lysed in a small volume (1–2 ml/dish) by aspirating the buffer and scraping with a rubber policeman; the lysate was collected and frozen until needed.

Beta-adrenergic receptor prelabeling. Lysates (6–7 mg of protein) from cells incubated in the presence of normal or heavy isotope-labeled amino acids were thawed and diluted to 10 ml with buffer A (145 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris, pH 7.4 at 37°). Receptors were prelabeled by incubating the membranes at 37° for 1 hr with the radiolabeled antagonist ^{125}I-CYP (80–100 pM, final concentration). All subsequent manipulations were carried out at 4°. After the 1-hr incubation, the concentration of free ^{125}I-CYP was reduced by three consecutive 20-min centrifugations at 45,000 × *g*. The pellet was resuspended by sonication in 8 ml of buffer A after each centrifugation. ^{125}I-CYP was prepared from (−)-cyanopindolol by iodination in the presence of chloramine-T as has been described previously for (−)-pindolol (16).

Solubilization. The prelabeled *beta*-receptors were extracted from the membrane by resuspending the final pellet in 1 ml of buffer A (pH 7.4 at 4%) containing 0.25% (w/v) Lubrol PX and incubating the sample on ice for 1 hr, vortexing periodically. The sample was then centrifuged at 40,000 rpm (177,000 × *g*) for 1 hr in a Beckman 42.1 rotor and a Beckman model L8-70 ultracentrifuge. The supernatant was carefully collected and applied to an 18-cm Sephadex G-50 column (i.d. = 8 mm). The column was eluted with a solution of 0.15% Lubrol PX in buffer A and the void volume containing the soluble prelabeled receptor was collected.

Density gradient ultracentrifugation. Continuous 3.5-ml sucrose density gradients were formed at 4° using an ISCO model 570 gradient former. All gradients contained 145 mM NaCl, 5 mM $MgCl_2$, and 20 mM Tris (pH 7.4 at 4°) in water. The linear gradients consisted of 5–15% (w/v) sucrose containing 0.15% Lubrol PX. Soluble ^{125}I-CYP-labeled receptor preparations were carefully layered (600 μl/gradient) on top of the gradients and centrifuged for 20 hr at 57,000 rpm (400,000 × *g*) in a Beckman SW-60 rotor and model L8-70 refrigerated ultracentrifuge. Fractions were collected from the top of each gradient using an ISCO model 568 fractionator at a flow rate of 0.375 ml/min and an ISCO model 1850 fraction collector (3 drops/fraction). The fractions were then counted in a gamma counter. The data obtained after gradient centrifugation are expressed as amount of ^{125}I-CYP bound per fraction. All of the radioactivity that migrated into the gradient apparently represents *beta*-receptor complexes since prelabeling with ^{125}I-CYP in the presence of 100 μM isoproterenol or 100 nM propranolol resulted in no radioactivity appearing in the gradient fractions.

Receptor-binding assays. Incubations for membrane binding assays were at 37° for 90 min with ^{125}I-CYP (0.2–70 pM) in a 1-ml final volume containing 145 mM NaCl, 5 mM $MgCl_2$, and 20 mM Tris, pH 7.4. The binding assay was terminated by filtration over glass fiber filters as

described previously (7). Nonspecific binding (binding in the presence of 100 μM isoproterenol) was <5% of the total counts retained by the filter.

RESULTS

As we have previously reported (3, 6, 7), incubation of confluent 1321N1 cells with isoproterenol for 24 hr resulted in a greater than 90% loss of *beta*-adrenergic receptors measured in cell homogenates with the lipophilic *beta*-adrenergic receptor antagonist ^{125}I-CYP (Fig. 1). Replacement of the growth medium with agonist-free medium resulted in recovery of *beta*-receptor levels to a specific activity similar to that of control cultures. This recovery process occurred whether the agonist-free medium consisted of normal or heavy amino acids (Fig. 1). A small decrease in the rate of *beta*-receptor recovery occurred subsequent to transfer to agonist-free medium containing heavy amino acids (Fig. 1).

The decrease in ^{125}I-CYP binding that occurred as a result of incubation of cells with isoproterenol was due to a decrease in *beta*-receptor number with no change occurring in K_d (3, 6, 7; and data not shown). As illustrated in Fig. 2, the binding affinity for ^{125}I-CYP of receptors recovering in the presence of heavy amino acids was similar to that for receptors recovering in the presence of normal amino acids. Thus, postconfluent 1321N1 cell cultures readily tolerate heavy amino acid-containing medium, and the recovery of down-regulated receptors in this medium apparently occurs with properties analogous to the process of recovery in normal growth medium.

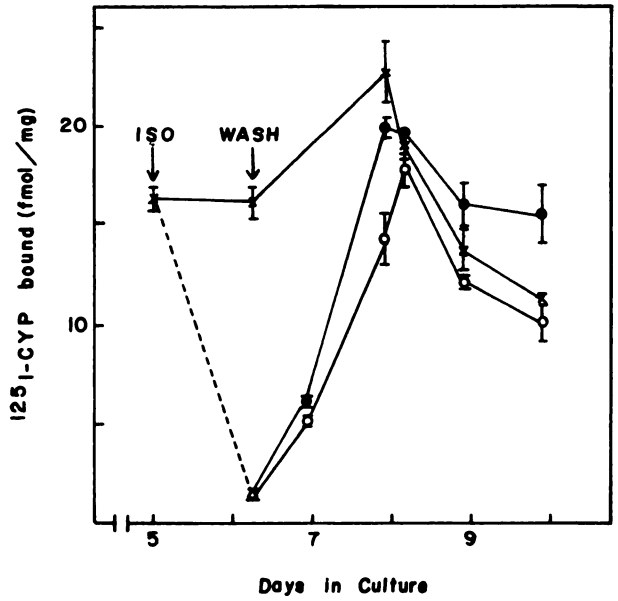

FIG. 1. *Recovery of beta-receptors in normal or heavy amino acid-containing medium.*

Postconfluent 1321N1 cell cultures were incubated for 30 hr with 1 μM isoproterenol (*ISO*). The cells were washed free of agonist and incubated for the indicated times in medium containing normal (●) or heavy (○) amino acids. ^{125}I-CYP was utilized to measure *beta*-receptors in lysates as described in Materials and Methods. *Beta*-receptor levels in control cultures (×) were also measured. These data are presented as mean ± standard deviation of triplicate dishes and are representative of two similar experiments.

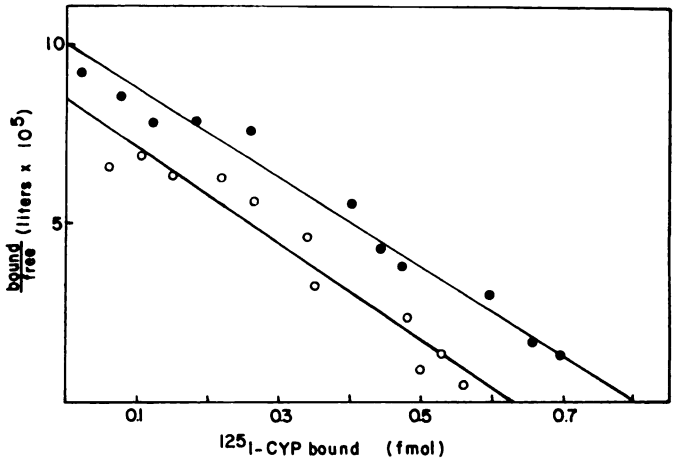

FIG. 2. *Scatchard analysis of ^{125}I-CYP binding to "normal" or "heavy" beta-receptors*

Postconfluent cultures were incubated with 1 μM isoproterenol for 30 hr and allowed to recover *beta*-receptors in agonist-free medium containing normal (●) or heavy (○) amino acids for 60 hr. The cells were lysed hypotonically and the specific binding of ^{125}I-CYP was measured as a function of ligand concentration (0.2–70 pM) at a protein concentration of approximately 50 μg/assay. The K_d values for ^{125}I-CYP in "normal" and "heavy" samples were 8.0 and 7.5 pM, respectively. The average K_d values from three independent experiments were 9.1 ± 0.6 pM for "normal" and 6.8 ± 0.4 pM for "heavy" *beta*-adrenergic receptors.

The principal goal of this study was to determine if *beta*-receptors that recover subsequent to long term incubation with catecholamines are a product of *de novo* synthesis. If the blockade of receptor recovery by cycloheximide previously observed (7) in postconfluent cultures was a result of effects of the protein synthesis inhibitor on the expression of another protein necessary for *beta*-receptor expression, then, in analogy with the situation in preconfluent cultures, new *beta*-receptor synthesis would be unnecessary to recover full binding activity in postconfluent cultures. Thus, the mass of *beta*-receptors recovering in heavy amino acid medium should be no different from that occurring in normal amino acid-containing medium. Conversely, if the effects of cycloheximide were explained by the necessity of *beta*-receptor protein synthesis per se for the recovery process, then *beta*-receptors recovered in heavy amino acid-containing medium should be of greater mass than those recovering in normal growth medium. Thus, 1321N1 cells were incubated with isoproterenol for 24 hr, the cultures were washed free of agonist, and incubation continued for another 60 hr in the presence of normal or heavy amino acid-containing growth medium. The cells then were lysed, and *beta*-receptors were labeled with ^{125}I-CYP, solubilized, and subjected to sucrose density gradient centrifugation as described in Materials and Methods. As illustrated in Fig. 3, the receptors that recovered in heavy amino acid-containing medium exhibited an increased mass compared to those recovering in normal growth medium. Figure 4 illustrates that the difference in receptor mass was observed with 10 separate preparations. Furthermore, when lysates from the two types of preparations were mixed in a 1:1 ratio, ^{125}I-CYP-labeled *beta*-receptors migrated with a larger peak width

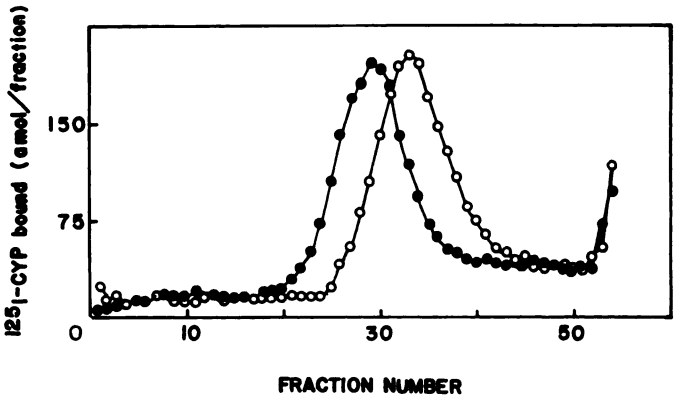

FIG. 3. *Resolution of solubilized "normal" and "heavy" beta-receptors on sucrose density gradients*

Postconfluent cultures were incubated with isoproterenol and allowed to recover in normal (●) or heavy (○) amino acid medium as described in Fig. 1. *Beta*-receptors in lysates from these cultures were prelabeled with ^{125}I-CYP and extracted with Lubrol-PX (see Materials and Methods). The soluble radiolabeled *beta*-receptors were layered on separate sucrose density gradients and centrifuged as described in the text. Fractions were collected and counted in a gamma counter. The top of the gradient is to the left. These data, expressed as attomoles per fraction, are representative of nine similar experiments.

to height ratio than was observed with either preparation alone.

DISCUSSION

The density shift method represents a powerful approach for the study of the regulation of synthesis and degradation of cellular proteins. This is particularly true for membrane receptors for neurotransmitters and hormones which are often present in the plasma membrane at a level as low as 1000–5000 molecules/cell. Thus, analysis of receptor metabolism based on the purification of receptor protein synthesized from radioactive amino acids is fraught with difficulties. Furthermore, the lack of generally available specific antibodies for most membrane receptors excludes this potentially fruitful approach for the study of receptor turnover. Based on the elegant studies of Fambrough and coworkers regarding acetylcholine receptors (12, 13), the density shift method provides a straightforward, unambiguous approach for study of receptor synthesis and degradation. This technique has recently been applied to the study of various aspects of insulin receptor turnover by Lane and coworkers (14, 15). Success with this method is primarily dependent on: (a) tolerance of the cells under study to the heavy amino acid containing medium; (b) availability of a protein-specific technique for labeling the receptor; and (c) availability of a means for solubilization of the receptor in a monodispersed form that can be separated on the basis of its mass from receptors synthesized from normal amino acids.

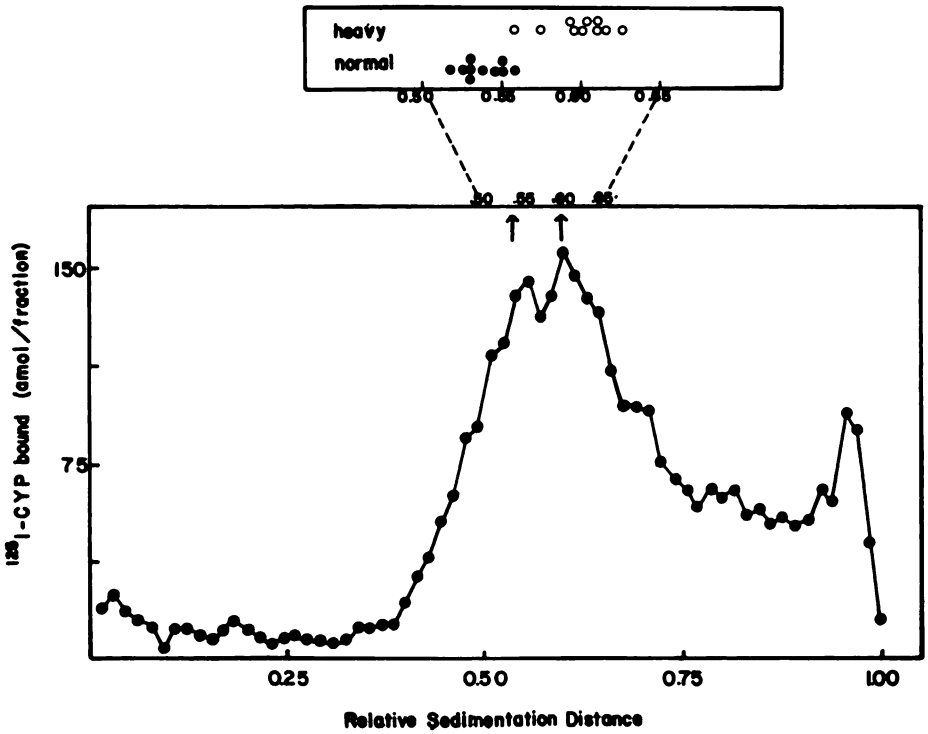

FIG. 4. *Gradient profile of a "mixed" normal and heavy beta-receptor preparation*

Beta-receptors recovered in normal or heavy amino acid medium were prelabeled and extracted with Lubrol-PX as described (see Fig. 1 and Materials and Methods). These two populations of soluble radiolabeled *beta*-receptors were mixed in a 1:1 ratio after elution from a Sephadex G-50 column, and layered on a single sucrose density gradient. The gradient was centrifuged and fractionated, and the fractions were counted as described in the previous figure. The data are plotted as attomoles per fraction versus the relative sedimentation distance into the gradient (fraction number/total number of fractions). The scale at the top has been expanded to show the distribution of peak activity from 10 individual gradients for both normal (●) and heavy (○) *beta*-adrenergic receptors. The average (± standard error of the mean) sedimentation positions (indicated by the arrows) for these two receptor populations were 0.538 ± 0.004 and 0.598 ± 0.006, respectively.

Although 1321N1 cells did not respond well to the heavy amino acid-containing growth medium in preconfluent culture conditions,[5] they tolerated this medium well under postconfluent culture conditions. Clearly, the heavy amino acid medium did not have a deleterious effect on the extent of *beta*-receptor recovery (Fig. 1) or on the functional properties of the receptor; only a slight reduction in the rate of recovery of receptors was observed in the presence of heavy amino acids. The availability of a highly selective, high specific activity, slowly dissociating radioligand for the *beta*-receptor (^{125}I-CYP) made the current study feasible. Essentially no non-*beta*-receptor binding of this radioligand occurs under the conditions used in these experiments, and negligible dissociation of ^{125}I-CYP-*beta*-receptor complexes occurs during maintenance of Lubrol-solubilized preparations at 4° for 2–3 days. As has been previously shown (17), *beta*-receptors from mammalian sources solubilized in Lubrol-PX exhibit a molecular size (M_r = 65,000–75,000) consistent with the minimal molecular weight determined for the purified functional receptor (18). Similar analysis of the sucrose density gradient centrifugation properties of the Lubrol-solubilized *beta*-receptor from 1321N1 cells revealed an estimated M_r = 70,000.

The conditions used in this study have been optimized for the separation of normal and heavy amino acid-containing *beta*-receptors on sucrose-H_2O and sucrose-D_2O gradients. Although we have not yet applied this methodology for the determination of values for the rates of *beta*-receptor synthesis and degradation, the extent of separation of heavy from normal receptors on these gradients should make direct determination of rates possible. We have not systematically evaluated the usefulness of other types of gradients for the separation of heavy and normal *beta*-receptors.

Our initial approach in the use of the density shift method was to use digitonin as the detergent for receptor solubilization. However, digitonin displays properties that make it unsuitable for this type of study. First, the percentage of total receptors solubilized by digitonin was low (<30%) compared to that effected with Lubrol PX (75–80%). Moreover, detergent binding and/or insertion of ligand-receptor complexes into digitonin micelles result in the generation of a species that migrates with much greater apparent size during sucrose density gradient centrifugation.[6] Previous studies with digitonin-solubilized *beta*-receptors also have indicated that the apparent size in the presence of digitonin (19, 20) is much greater than the size in the presence of Lubrol PX. Probably due to this rather large detergent-dependent increase in the apparent size of both normal and heavy *beta*-receptors, the increase in mass resulting from incorporation of heavy amino acids into the receptor protein was not detectable for digitonin-solubilized receptors by sucrose density gradient centrifugation in either H_2O or D_2O.[6] Thus, in our hands, digitonin is not a suitable detergent for studies of *beta*-receptor turnover using the density shift method.

The purposes of this study were 2-fold. First, we desired methodology for unambiguously measuring *beta*-receptor synthesis. The data indicate that in postconfluent 1321N1 cell cultures the density shift method can be used for this purpose.[5] Second, it was important to determine whether *beta*-receptors recovering after down-regulation in confluent cultures were the products of *de novo* synthesis. As is clearly demonstrated (in Fig. 3), this is the case. Receptors that reappear after down-regulation exhibit an increase in mass. This represents the first unequivocal demonstration of *beta*-receptor resynthesis as a step in recovery from catecholamine-induced desensitization. This mechanism apparently distinguishes between the properties of *beta*-receptor regulation in post-versus preconfluent cultures, since in the latter case, receptors can recover in the presence of cycloheximide (7) after down-regulation. Whether this difference in the properties of expression of a membrane protein in pre- versus postconfluent cultures is a general phenomenon or is restricted to the *beta*-adrenergic receptor of 1321N1 astrocytoma cells is yet to be determined.

[5] Numerous potential approaches for using the density shift methodology in preconfluent 1321N1 cell cultures proved unsuccessful. Cells in these cultures underwent dramatic morphological changes and ceased division within 24–48 hr after application of the heavy amino acid-containing medium. No evidence of toxicity was observed in postconfluent cultures.

[6] R. C. Doss, G. L. Waldo, J. Perkins, and T. K. Harden, unpublished observations.

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