Recovery of β-Adrenergic Receptors following Long Term Exposure of Astrocytoma Cells to Catecholamine

ROLE OF PROTEIN SYNTHESIS

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As a part of the process of agonist-induced desensitization, 1321N1 human astrocytoma cells lose up to 95% of their β-adrenergic receptors, as detected by [125I]hydroxybenzylpindolol ([125I]HYP) binding, after 12-24 h of exposure to isoproterenol. In preconfluent cultures the loss of β-receptors is completely reversible upon removal of isoproterenol, with receptor levels reaching 100% of control levels within 48-72 h. Addition of cycloheximide (5 μg/ml) upon removal of agonist does not prevent the recovery of receptors. After an initial 4-h lag, receptors accumulate in the presence of cycloheximide until the same receptor level is reached that was present at the onset of desensitization. Confluent cultures, which have a reduced number of receptors per cell, recover β-receptors to only 60 to 70% of control levels following removal of isoproterenol. In addition, cycloheximide blocks the recovery of receptors in these cultures. The effects of cycloheximide on the accumulation of receptors during cell growth suggest that receptors are stable in preconfluent cultures and that turnover only occurs later when cultures are confluent. The data also indicate that long term exposure of cells to catecholamine results in a form of the β-adrenergic receptor that is undetectable by [125I]HYP binding but, nonetheless, retains its primary amino acid structure. The undetectable receptors appear to be retained until agonist is removed, whereupon they become detectable by [125I]HYP binding with a t1/2 of about 36 h in the presence of cycloheximide.

Catecholamine-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase occurs by a multistep process in 1321N1 astrocytoma cells (1-3), S49 lymphoma cells (3), frog erythrocytes (4), and certain C6 glioma cells (5). An early event in the process of desensitization results in the "uncoupling" of the β-adrenergic receptor from adenylate cyclase. In 1321N1 astrocytoma cells this uncoupling is expressed as a selective loss of responsiveness of adenylate cyclase to catecholamines with no alteration in receptor number as assessed by [251H]iodohydroxybenzylpindolol ([251H]I) binding. The mechanism leading to agonist-induced alteration of β-receptor binding properties and the ultimate cellular destination of the altered receptors remain unclear. The present study examines the fate of β-adrenergic receptors lost during chronic exposure of 1321N1 astrocytoma cells to catecholamines.

EXPERIMENTAL PROCEDURES

Materials—All cell culture materials were obtained from commercial sources as previously reported (11). (-)-Isoproterenol-HCl, cycloheximide, ascorbic acid, and Heps were from Sigma. Carrier-free Na[125I] was purchased from Amersham, and 1-[4,5-3H]leucine (30-50 Ci/mmol) was from ICN. Hydroxybenzylpindolol was a generous gift from Drs. D. Hauser and R. Berthold of Sandoz (Basel). All other reagents used were of analytical grade.

Cell Culture Conditions—The 1321N1 human astrocytoma cells were maintained in Dulbecco’s modification of Eagle’s medium supplemented with 5% fetal bovine serum in an atmosphere of 95% air and 5% CO2 at 37 °C in a humidified incubator. Cells were grown in the absence of antibiotics, and the medium was replenished every 2-3 days. Cells were subcultured by detaching the monolayer with a Heps-buffered (pH 7.8) 0.05% trypsin solution. The trypsinization was previously shown to have no effect on β-adrenergic receptor number (11). In all experiments, β-adrenergic receptor number was assessed in membrane fractions derived from cells grown on 100-mm tissue culture dishes (Falcon). All cells were seeded from 150-mm dishes that contained confluent cells which were at least 7 days old.

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1 The abbreviations used are: Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [125I]HYP, [125I]iodohydroxybenzylpindolol.
These cells are termed "long term" cultures and exhibited less than optimal responsiveness to catecholamines (11).

(±)-Isoproterenol-HCl was added to all appropriate incubations in a solution that contained sodium ascrobate (final concentration, 1 mM) as an antioxidant. Fresh isoproterenol was added every 6-8 h during a typical 12-24 h incubation with the drug. Sodium ascrobate (final concentration, 1 mM) alone was added to control dishes. All drugs were prepared as fresh solutions, and fresh growth medium was added to all the dishes prior to the addition of the drug.

Broken Cell Preparations—Growth medium was aspirated from the dishes, and each cell sheet was rinsed twice with 5 ml of ice-cold lysing buffer which consisted of 1 mM Tris (pH 7.8 at 4 °C) and 0.5 mM MgCl2. The cells were then swollen in 5 ml of lysing buffer for 10 to 15 min and lysed by scraping the dishes with a rubber spatula. Each suspension of lysed cells was centrifuged for 10 min at 34,000 x g in a Beckman J21C centrifuge at 4 °C. The resultant pellet was resuspended in 0.3 ml of 0.25 M sucrose and 5 mM MgCl2 in 50 mM Tris (pH 7.8 at 4 °C) and stored at -70 °C until assayed. Samples stored in this manner showed no significant change in β-adrenergic receptor binding properties during several months of storage.

β-Adrenergic Receptor Assay—Hydroxybenzylpindolol was iodinated, and the radioactive product, 125I-HYP, was purified chromatographically and used in assessing β-adrenergic receptor density as previously described in detail (12-14). In most experiments 125I-HYP (8,000,000 dpm, 70 pm) was incubated with tissue (15 to 55 μg of protein), 20 mM Tris, pH 7.5, 2 mM MgCl2, 1 mM sodium ascrobate, and 140 mM NaCl in a volume of 0.25 ml. After a 30-min incubation at 37 °C, 10 ml of 140 mM NaCl in 10 mM Tris buffer (37 °C, pH 7.5) was added to each assay tube, and the samples were rapidly filtered using 25-mm glass fiber filters (Schleicher and Schuell #30). Each filter was washed with an additional 10 ml of 10 mM Tris, pH 7.5, at 37 °C. Nonspecific binding was defined as the amount of 125I-HYP bound in the presence of 100 μM isoproterenol. Specific binding represented 80 to 95% of the radioactivity retained by the filters. In some experiments, tissue was incubated with various concentrations (5 to 200 pm) of 125I-HYP, and the amount of radioligand specifically bound at each concentration was determined as described above. These data were analyzed by the method of Scatchard (15) to provide values for the number of receptors and the dissociation constant of 125I-HYP.

Protein Assay—Protein concentration was determined by the method of Lowry et al. (16) using bovine serum albumin as a standard.

Data Presentation and Analysis—Assays were routinely carried out in duplicate or triplicate, and, in all experiments, three samples were assayed for each data point. Data are presented as the mean ± standard error of specifically bound 125I-HYP. The number of times a particular experiment was repeated is provided in the figure legends.

RESULTS

Recovery of β-Adrenergic Receptors Following Long Term Exposure of Cultures to Isoproterenol—As we have previously reported (11) the expression of β-adrenergic receptors during growth of 1321N1 cells in culture (Fig. 1) was dependent on cell density. The specific activity of receptors increased markedly during the first 60 h in culture, reaching a maximal level within 3 days (Fig. 1, upper panel). Following 2 days at this maximal level, the number of receptors per cell declined as cell density increased past confluency. The decline in specific activity of receptors is a reflection of increases in the number of cells per culture at a time (i.e. > 100 h postsubculture) when receptor number per dish remains constant (Fig. 1, upper and lower panels).

Incubation of cells in the presence of 0.1 μM isoproterenol2

2 The concentration of isoproterenol (0.1 μM) used in these experiments was sufficient to produce maximal loss of β-receptors with no effects on cell viability. This concentration of isoproterenol produces maximal elevation of cyclic AMP levels in these cells (1).
for 12 h results in a marked reduction in the number of receptors with no apparent alteration in the $K_d$ for $^{125}$I-HYP as determined by Scatchard analysis (Fig. 2). Fig. 1 demonstrates that cultures which had lost 90-95% of their $\beta$-adrenergic receptors during a 12-h incubation with isoproterenol were able to recover receptors after removal of the isoproterenol. The pattern and extent of recovery of $\beta$-adrenergic receptors showed a dependence on cell density. Thus, after a 12-h exposure of sparse cultures (48 h postsucubculture) $\beta$-adrenergic receptors accumulated rapidly upon removal of isoproterenol, reaching 100% of control receptor number within 48-72 h. More dense cultures (120 h postsucubculture) recovered receptors to only 60-70% of control levels following removal of isoproterenol.

**Fig. 3.** Effect of cycloheximide on the expression of $\beta$-adrenergic receptors during growth of 1321N1 cells. Cells were seeded as described in Fig. 1 and treated with cycloheximide (final concentration, 5 $\mu$g/ml) at 48 h ($\bullet$), 72 h ($\circ$), and 120 h ($\triangle$) postsucubculture. Fresh growth medium and cycloheximide were added 48 h after the initial addition. Control cultures ($\Box$) were not treated with cycloheximide. Membrane fractions were isolated at the indicated times and assayed for $\beta$-adrenergic receptors as described in Fig. 1 and under "Experimental Procedures." The data are representative of two similar experiments.

**Table I**

**Effect of cycloheximide on 1321N1 cell growth**

| Time (h) | Control | Isoproterenol washedout & cycloheximide | Isoproterenol washedout & cycloheximide | Cycloheximide washedout |
|----------|---------|----------------------------------------|----------------------------------------|------------------------|
| 0        | 280,000 |                                        |                                        |                        |
| 24       | 249,660 |                                        |                                        |                        |
| 48       | 448,780 |                                        |                                        |                        |
| 60       | 569,990 | 521,200                                | 532,760                                |                        |
| 72       | 722,280 | 685,620                                | 525,060                                |                        |
| 84       | 935,600 | 814,700                                | 517,480                                | 510,521                |
| 108      | 1,416,160 | 1,261,800                           | 492,520                                | 674,460                |
| 132      | 2,805,600 | 2,272,360                           | 443,760                                | 1,171,320              |
| 156      | 4,235,380 | 3,877,840                           | 388,560                                | 1,736,480              |
| 180      | 7,015,360 | 6,584,420                           |                                        | 3,460,000              |

**Fig. 4.** Effect of cycloheximide on recovery of $\beta$-adrenergic receptors following long term exposure of preconfluent cultures to isoproterenol. Cells were seeded as described in Fig. 1. (e)-Isoproterenol (final concentration, 0.1 $\mu$M) was added to 48-h cultures with fresh drug added 6 h later. After a 12-h incubation with isoproterenol (ISO), the dishes were washed with agonist-free medium as described in Fig. 1, and 10 ml of Dulbecco's medium plus 5% fetal calf serum was added. Cycloheximide (CHX) (final concentration, 5 $\mu$g/ml) was added to some of the cultures ($\Delta$, $\bigcirc$), while other cultures ($\Box$) were allowed to recover in the absence of cycloheximide. Control cultures ($\bigcirc$) received only 1 mM ascorbate as described under "Experimental Procedures." After 24 h in the presence of cycloheximide some of the cycloheximide-treated cultures were washed free of cycloheximide ($\bigcirc$) and allowed to recover in cycloheximide-free medium. Fresh cycloheximide and medium were added 48 h after the initial addition of the drug to the cycloheximide-treated cultures. Membrane fractions and assays were performed as described under "Experimental Procedures." The data are representative of three similar experiments.

**Effect of Cycloheximide on the Expression of $\beta$-Adrenergic Receptors in Culture**—In an attempt to estimate $\beta$-adrenergic receptor turnover rates, the effect of cycloheximide on receptor content was examined. Addition of cycloheximide (final concentration, 5 $\mu$g/ml) to preconfluent 1321N1 astrocytoma cultures (i.e. 48 h and 72 h postsucubculture) produces a marked
inhibition of the accumulation of β-adrenergic receptors observed during the initial stages of culture (Fig. 3). The concentration of cycloheximide (5 μg/ml) employed was found to cause a greater than 85% inhibition of [3H]leucine incorporation into 1321N1 cellular protein (data not shown) and a total inhibition of cell growth (Table I). Receptor levels appeared to remain relatively constant throughout the incubation with cycloheximide; however, a slight increase in specific activity was observed. This appears to be due to a decrease in total protein per culture dish. Addition of cycloheximide to confluent cultures (i.e. >100 h postsubculture) resulted in a gradual decrease in β-adrenergic receptor levels (Fig. 3) with time. Although cycloheximide inhibited cell division, cells were able to recover and grow following a 24-h (Table I) or 48-h (data not shown) exposure to the drug. It appears, therefore, that chronic exposure to cycloheximide (5 μg/ml) does not affect cell viability.

**Effect of Cycloheximide on the Recovery of β-Adrenergic Receptors in Preconfluent Cultures**—Preconfluent cultures (48 h postsubculture) were incubated with 0.1 μM isoproterenol for 12 h such that 90% of the receptor sites were lost (Fig. 4). Isoproterenol was subsequently washed out and agonist-free medium added. Addition of cycloheximide (5 μg/ml) immediately following the removal of isoproterenol completely inhibited the recovery of receptors for the first 4 h (Fig. 5). However, after this initial lag receptors reappeared even in the presence of cycloheximide albeit at a 50% reduction in rate (Figs. 4 and 5). This reduction in rate is probably due to the fact that cycloheximide inhibits cell growth. Receptor content continued to increase in the presence of cycloheximide until the cultures expressed the same number of receptors that were present at the time isoproterenol was initially added (48 h post-subculture). When cycloheximide was removed, either 24 or 48 h after its addition, the β-adrenergic receptor levels rapidly increased to control levels (Fig. 4).

Throughout the incubation with cycloheximide, protein synthesis was inhibited between 85-95% as assessed by [3H]leucine incorporation into trichloroacetic acid-precipitable material. Cell growth also was completely blocked by cycloheximide as shown in Table I. The time coordinates of Table I correspond to the conditions in Fig. 4. Data in Table I clearly show that addition of cycloheximide at 60 h postsubculture blocks cell growth; however, normal growth of cells resumed if cycloheximide was washed out 24 h (Table I) or 48 h (not shown) after its addition. Although 10-20% of the cells were lost during a 96-h exposure to cycloheximide, the majority of the cells remained attached to the culture dish and appeared to retain their normal morphology.

The initial 4-h lag observed (Figs. 4 and 5) in the presence of cycloheximide was not readily apparent in cells which were allowed to recover in the absence of cycloheximide. However, it should be noted that the rate of receptor accumulation in cells allowed to recover in the absence of cycloheximide includes both the rate of receptor synthesis due to cell growth and the rate of recovery of lost β-receptors. If the contribution of cell growth to the rate of receptor accumulation is subtracted from the observed rate in cultures allowed to recover in the absence of cycloheximide, a 4-h lag is apparent. Thus, it seems reasonable to conclude that receptor synthesis due to cell growth masks the lag in recovery of lost receptors in cycloheximide-free cultures and that the lag is not an artifact.
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of cycloheximide treatment.
Preconfluent cultures that expressed nearly maximal specific activity of β-adrenergic receptors (60 h postsubculture) and had lost greater than 90% of their receptors after a 12-h exposure to isoproterenol were still able to recover a full complement of receptors in the presence of cycloheximide following removal of isoproterenol (data not shown). In addition, the recovery of receptors after 24 h of exposure to isoproterenol is as rapid and as complete as after 12 h of exposure to agonist (data not shown).

β-Adrenergic receptors that had recovered in the presence of cycloheximide following removal of isoproterenol displayed an affinity ($K_d = 26$ pm) for $[^3]HYP$ similar to that of receptors that had recovered without cycloheximide ($K_d = 23$ pm) and that of control cells ($K_d = 28$ pm) (Fig. 2). Thus, the increase in $[^3]HYP$-binding sites in the presence of cycloheximide following removal of isoproterenol was not due to an increase in affinity of the receptor for $[^3]HYP$ but was in fact due to an increase in receptor number.

Effect of Cycloheximide on the Recovery of β-Adrenergic Receptors in Confluent Cultures—Since 1321N1 cells show a marked reduction in β-receptor number per cell after cell contact occurs, it was proposed that confluent cultures may have a reduced capability of synthesizing new receptors, or alternatively, an increased degradative capacity (11). Therefore, it was of interest to determine the effects of cycloheximide on the recovery of β-adrenergic receptors following long term exposure of confluent cultures to isoproterenol. Confluent cultures (120 h postsubculture) were incubated with 0.1 μM isoproterenol for 12 h such that 90% of the receptors were lost (Fig. 6). In contrast to preconfluent cultures, the recovery of β-receptors following removal of isoproterenol was blocked by cycloheximide in these cultures. Fig. 6 also shows that confluent cultures could not recover a full complement of receptors even in the absence of cycloheximide, with receptor levels reaching only 70% of control levels.

Discussion
Reversal of catecholamine-specific desensitization appears to occur by at least two different processes. The initial uncoupling reaction is readily reversible ($t_{1/2} = 7$ min) upon removal of agonist (3). Furthermore, recovery from the uncoupled form of the receptor to the form capable of coupling to adenylate cyclase is complete provided no receptor loss has occurred. In contrast, our earlier studies indicated that recovery of lost receptors was negligible (3). It was suggested that loss of receptors was essentially irreversible and might be a result of receptor internalization and degradation (1, 3, 6). The present studies indicate that under appropriate growth conditions the β-receptors lost to detection by $[^3]HYP$ binding can in fact regain binding activity. The fact that lost receptors are fully recovered in the presence of cycloheximide suggests that the primary structure of the receptor is conserved, and, therefore, degradation is not involved in agonist-induced receptor loss in preconfluent cultures.

The effects of cycloheximide on the accumulation of receptors in culture reflect changes in receptor accumulation during growth. The absence of receptor loss in preconfluent cultures during chronic exposure to cycloheximide (Fig. 3) suggests that receptor turnover is negligible in such cultures. In analogy to insulin receptor turnover in 3T3-L1 mouse adipocytes (17), it is possible that a protein of very short half-life which is necessary for the normal degradation of β-receptors is lost during the incubation with cycloheximide. Although we cannot completely rule out this possibility, preliminary observations of β-receptor turnover in the presence of tunicamycin, an inhibitor of dolichol pyrophosphate-mediated protein glycosylation (18), also indicate essentially no receptor turnover.

Although tunicamycin (0.1 μg/ml) inhibits protein synthesis by only 15%, receptor accumulation is completely blocked, and receptor number per dish remains constant throughout chronic exposure to the drug. The small increase in specific activity during incubation of preconfluent cultures with cycloheximide (Fig. 3) probably does not represent presynthesized receptors in transit from intracellular pools to the plasma membrane, as has been reported for the acetylcholine receptor (19). Instead, the increase can be accounted for by a loss in total cellular protein during a time when receptor number per dish remains constant.

In contrast to the situation in preconfluent cultures, receptor turnover apparently does take place in confluent cultures as indicated by the fact that receptor loss occurs in the presence of cycloheximide (Fig. 3). One might speculate that cell contact could induce a degradation process for β-receptors which apparently is absent in preconfluent cultures. Evidence from this study that suggests degradation of the β-receptor takes place only in confluent cultures includes the following.

(i) Confluent cultures are not capable of recovering lost receptors in the presence of cycloheximide following exposure to catecholamines, whereas lost receptors in preconfluent cultures recover in the presence of cycloheximide to the level of receptors present at the onset of desensitization. (ii) In the absence of cycloheximide, confluent cultures which have lost 90% of their receptors can recover only to 70% of control levels following removal of isoproterenol. On the other hand, preconfluent cultures recover a full complement of receptors following washout of isoproterenol. (iii) Receptor number and specific activity in confluent cultures show a slight decrease with chronic exposure to cycloheximide, whereas preconfluent cultures show no receptor turnover. The best current interpretation of our data is that β-adrenergic receptor degradation is significant only in late or confluent cultures.

Our data suggest that receptor recycling occurs in 1321N1 cells. The key questions that remain to be answered concern the mechanisms leading to the loss of receptor-binding sites and the cellular location of the lost receptors. In this regard a great deal is already known concerning the down regulation of peptide hormone receptors. Several peptide hormones have been shown to be internalized after their binding to cell surface receptors (7–10, 20–22). However, there have been conflicting reports concerning the fate of the internalized hormone-receptor complexes. For example, once internalized, the epidermal growth factor (EGF)-receptor complex fuses with lysosomes where the entire complex is degraded (20, 23, 24). In contrast, the insulin-receptor complex does not appear to be degraded as a single entity. Krupp and Lane (25) have suggested that insulin degradation and receptor degradation are separable processes. The results of some investigators suggest that certain receptors are spared from degradation entirely and may be recycled back to the cell surface (see Ref. 26).

In addition to the different mechanisms proposed for the regulation of EGF and insulin receptor levels, there also have been conflicting reports concerning the regulation of β-receptors. In agreement with the results in our study concerning β-receptor regulation in preconfluent 1321N1 cultures, an inability of cycloheximide to prevent the restoration of sensitivity to catecholamines has been observed in frog erythrocytes (27) and in Ehrlich ascites cells (28). However, cycloheximide inhibited the recovery of catecholamine-stimulated adenylate cyclase activity in BHK cells after desensitization with epinephrine (29). This latter finding may be analogous...
to our present observations that confluent 1321N1 cells are unable to recover lost β-receptors in the presence of cycloheximide. The discrepancies observed between different cells and between preconfluent and confluent 1321N1 cells probably reflect different mechanisms leading to agonist-induced loss of receptors.

At this point we can only speculate on the mechanisms for catecholamine-induced receptor loss and the fate of the lost receptors. There is no direct evidence for β-receptor internalization in 1321N1 cells, and in fact, enzymatic, reversible modifications (e.g. phosphorylation, dephosphorylation; methylation, demethylation) could explain our data. Previous reports from our laboratory have shown that agonist-induced "uncoupled" receptors do not co-migrate with plasma membrane marker enzymes on sucrose gradients (6). It was suggested that the uncoupled receptors might exist in cytoplasmic vesicles as a result of selective endocytosis similar to the process proposed for internalization of other hormone-receptor complexes (7–10). Such a mechanism has been implicated recently for loss of membrane β-receptors during desensitization of frog erythrocytes (30, 31). Investigations are currently underway to define the mechanisms for the regulation of β-receptor synthesis and processing in 1321N1 astrocytoma cells.

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