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Effects of Tunicamycin on the Expression of β-Adrenergic Receptors in Human Astrocytoma Cells during Growth and Recovery from Agonist-Induced Down-Regulation

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SUMMARY

Tunicamycin, which inhibits formation of asparagine-linked glycoproteins, caused a concentration-dependent blockade of β-adrenergic receptor (β-AR) accumulation in 1321N1 human astrocytoma cells during growth in culture. A concentration of tunicamycin (0.1 μg/ml) that inhibited receptor accumulation and [^{3}H]mannose or [^{3}H]glucosamine incorporation into glycoproteins by 90% had only a small effect (10%) on [^{3}H]leucine incorporation into protein, and reduced the rate of cell growth. Incubation in drug-free medium subsequent to treatment of 1321N1 cells with tunicamycin for 48 hr resulted in recovery of β-AR to control levels within an additional 48 hr. Exposure of cultures to isoproterenol (0.1 μM, 12 hr) caused an 80–90% loss of β-AR in both pre- and postconfluent cultures; β-AR recovered to control levels upon removal of isoproterenol. Although both tunicamycin and the protein synthesis inhibitor cycloheximide blocked β-AR accumulation during growth of 1321N1 cells, neither agent inhibited the appearance of β-AR during recovery from the down-regulated state in *preconfluent* cultures. However, cycloheximide, but not tunicamycin, blocked recovery of β-AR after isoproterenol-induced loss of receptors in *postconfluent* cultures. In a previous report (*Mol. Pharmacol.* **26:** 424–429, 1984), we provided direct evidence that recovery of β-AR from down-regulation in postconfluent cultures requires *de novo* synthesis of receptor protein. Thus, the results with tunicamycin are consistent with the idea that recovery of β-AR in postconfluent cultures requires the synthesis of new β-AR molecules, but as aglycoproteins that exhibit radioligand-binding characteristics similar to those of native glycoprotein β-AR.

INTRODUCTION

The β-AR[3] is a plasma membrane ectoprotein and a component of the hormone-sensitive adenylate cyclase system (1–3). Many of the ectoproteins of the mammalian cell plasma membrane have been shown to be glycoproteins (4); several lines of evidence suggest that the β-AR also is a glycoprotein. For example, detergent-solubilized β-AR can be bound to lectins (5–7) and eluted with an appropriate competing ligand (7). Also, treatment of detergent-solubilized β-AR with endoglycosidase F results in a marked reduction in apparent molecular weight as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5).

Incubation of target cells with β-AR agonists results not only in stimulation of adenylate cyclase but also in a desensitization of the response of the cells to catecholamines (8, 9). Such agonist-induced desensitization processes appear to involve rapidly reversible "uncoupling" reactions that selectively reduce responsiveness of adenylate cyclase to catecholamines (8–13), as well as more chronic changes that include "down-regulation" of cellular β-AR (9, 11, 14–18). These adaptive phenomena have been studied in detail primarily in erythrocytes and cultured cells (8, 9).

Examination of the properties of down-regulation of β-AR in this laboratory principally has been carried out with 1321N1 human astrocytoma cells in which exposure to isoproterenol leads to greater than 90% loss of β-AR with a $t_{1/2}$ of about 4 hr (11, 14). Following removal of isoproterenol, receptor number returns to control levels; the characteristics of the recovery process vary depending on whether pre- or postconfluent cultures are studied (14, 19). For example, cycloheximide, which completely

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[3] The abbreviations used are: β-AR, β-adrenergic receptor; HYP, hydroxybenzylpindolol; PIN, pindolol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modification of Eagle's medium; HPLC, high performance liquid chromatography.

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blocks β-AR accumulation in untreated, preconfluent cultures, has no effect on the extent of recovery of β-AR after agonist-induced down-regulation in preconfluent cultures; in contrast, it completely blocks recovery in postconfluent cultures. Thus, it was proposed (14) that synthesis of β-AR or of another protein was required for recovery of β-AR in postconfluent cells, whereas β-AR synthesis was not involved in the recovery process in preconfluent cells.

We report here the effects of tunicamycin on the expression of β-AR in 1321N1 cells during normal growth in culture and during recovery from catecholamine-induced down-regulation. Since tunicamycin blocks dolichol phosphate-mediated, asparagine-linked glycosylation of proteins (20), it seemed probable that this antibiotic would block the synthesis of β-AR. The results indicate that, whereas tunicamycin completely, but reversibly, blocks β-AR accumulation in growing cells, it has essentially no effect on the rate or extent of recovery of down-regulated β-AR in either pre- or postconfluent cells. The lack of effect of tunicamycin on recovery of β-AR in postconfluent cells is of interest since other observations (21) establish that *de novo* synthesis of β-AR occurs during recovery in such cultures.

EXPERIMENTAL PROCEDURES

Materials. All cell culture materials were obtained from commercial sources as previously reported (14, 19). Tunicamycin, (±)-isoproterenol HCl, cycloheximide, ascorbic acid, and Hepes were from Sigma. Carrier-free $Na^{125}I$, D-[6-^{3}H]glucosamine (20–40 Ci/mmol), and D-[2-^{3}H]mannose (10–20 Ci/mmol) were purchased from Amersham. L-[4,5-^{3}H] Leucine (30–50 Ci/mmol) was from ICN. Hydroxybenzylpindolol was a generous gift from Drs. D. Hauser and R. Berthold of Sandoz (Basel). Pindolol was a generous gift from Dr. G. Engel of Sandoz (Basel). All other reagents were of analytical grade.

Cell culture conditions. The 1321N1 human astrocytoma cells were maintained in DMEM supplemented with 5% fetal bovine serum in an atmosphere of 92% air and 8% CO_2 at 37° 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 Hepes-buffered (pH 7.8) 0.05% trypsin solution. In all experiments, cells were seeded from 150-mm dishes that contained confluent cells that were at least 7 days old. Receptor number was assessed in homogenates derived from cells grown on 60- or 100-mm culture dishes.

All drugs were prepared as fresh solutions, and fresh growth medium was added to the dishes prior to the addition of the drug. Tunicamycin or cycloheximide was readded every 48 hr along with fresh growth medium. Isoproterenol was added to cultures in ascorbic acid (1 mM, final concentration). Fresh agonist was added every 6 hr.

β-AR assay. Assays for β-AR content were performed on membranes obtained from lysed cells as previously described (14). Hydroxybenzylpindolol (22) or pindolol (23) was iodinated, and the radioactive products, ^{125}IHYP or ^{125}IPIN, were purified chromatographically. The same value for the number of receptors was obtained when either ^{125}IHYP or ^{125}IPIN was used as the radioligand. In most experiments ^{125}IHYP or ^{125}IPIN (80,000 dpm, 70 pM) was incubated with tissue (15–55 μg of protein), 20 mM Tris (pH 7.5), 2 mM $MgCl_2$, 1 mM sodium ascorbate, and 140 mM NaCl in a volume of 0.25 ml. After a 60-min incubation at 37° (for ^{125}IHYP) or 25°(for ^{125}IPIN), 10 ml of wash buffer consisting of 140 mM NaCl and 10 mM Tris (pH 7.5) was added to each assay tube, and the samples were rapidly filtered using 25-mm glass fiber filters (Schleicher and Schuell No. 30). Each filter was washed with an additional 10 ml of wash buffer. Nonspecific binding was defined as the amount of ^{125}IHYP or ^{125}IPIN bound in the presence of 100 μM isoproterenol. Specific binding represented 80–95% of the radioactivity retained by the filters. In some experiments, tissue was incubated with various concentrations (5–230 pM) of ^{125}IPIN or ^{125}IHYP, and the amount of radioligand specifically bound at each concentration was determined as described above. These data were analyzed by the method of Scatchard (24) to provide values for the number of receptors (B_{max}) and equilibrium dissociation constant (K_d) of the radioligand.

Analysis of the effects of tunicamycin on protein synthesis and glycosylation. Cells were subcultured (6000–9000 cells/cm^2) onto 60-mm dishes as described above. Forty-eight hr after subculture, fresh growth medium containing tunicamycin (usually 0.1 μg/ml, final concentration) was added. D-[2-^{3}H]Mannose and/or D-[6-^{3}H]glucosamine were used to assess protein glycosylation and L-[4,5-^{3}H]leucine was used to measure protein synthesis per se. In each case, radioactive compounds were added at a final concentration of 3 μCi/ml. In most experiments, the cells were labeled for 6 hr and subsequently washed three times with 2 ml of 20 mM Tris (pH 7.5 at 25°), 140 mM NaCl, and 5 mM $MgCl_2$. Labeled material was solubilized with 1.0 ml of 0.1 N NaOH, and the dish was washed with an additional 0.5 ml of 0.1 N NaOH which was combined with the initial 1.0 ml. Samples that had been labeled with [^{3}H]mannose or [^{3}H]glucosamine were incubated at 37° for 18 hr. This procedure hydrolyzes all serine- or threonine-linked oligosaccharides and thus allows quantitation of the incorporation of label into asparagine-linked glycoproteins (25). Labeled protein was precipitated by the addition of 2 ml of 10% trichloroacetic acid and the samples were centrifuged at 6000 × *g* for 10 min. The supernatant was discarded and the pellet was washed by solubilization in 1.0 ml of 0.1 N NaOH followed by the addition of 2 ml of 10% trichloroacetic acid. The samples were centrifuged again as described above and the supernatant was discarded. The pellet was resolubilized in 1.3 ml of 0.1 N NaOH and 1.0 ml of this material was used for determination of radioactivity. The remaining 0.3 ml was used for the determination of protein content by the method of Lowry *et al.* (26).

Purification of tunicamycin by HPLC. Since tunicamycin is a mixture of structurally related antibiotics (27), an attempt was made to purify the individual components with the idea that one of the components might be more selective in inhibiting β-AR accumulation. A Waters Associates model 6000A instrument equipped with a 254-nm detector (Waters Associates model 44) was employed for the HPLC purification of tunicamycin (27). Tunicamycin (60–100 μg) was injected onto a Bio-Sil ODS-10 column (4 × 250 mm; Bio-Rad) and was eluted at a flow rate of 1 ml/min using methanol:25 mM sodium acetate buffer, pH 5.0 (7:3, v/v). Five major peaks were collected and, after 10 runs (10 separate injections), the respective fractions were pooled and concentrated to dryness by rotary evaporation. The dried material was resuspended in 1.0 ml of 0.1 N NaOH. The quantity of material in each peak was determined by adsorption at 260 nm using an extinction coefficient of 9650 M^{-1} cm^{-1} and molecular mass of 870 g/mol. Each of the fractions inhibited [^{3}H]mannose incorporation into protein. The incorporation of [^{3}H]glucosamine also was inhibited (48–82%) by the HPLC fractions. In contrast, the fractions produced negligible effects on protein synthesis as measured by [^{3}H]leucine incorporation. None of the fractions alone was as effective as the complex tunicamycin mixture in inhibiting glycosylation during a 24- or 48-hr incubation. Similar results were obtained in experiments examining the effects of the individual components on β-AR accumulation. While each HPLC-purified component reduced receptor accumulation during a 24- or 48-hr incubation, none of the components was as effective as the complex mixture. In light of these results, all experiments presented in this study were carried out with the tunicamycin complex.

RESULTS

Relation of growth conditions and accumulation of β-AR in 1321N1 cells. When 1321N1 cells are subcultured from postconfluent cultures in the standard manner (19), they exhibit three characteristic stages of β-AR expression. During the first 24–48 hr of log phase growth, the

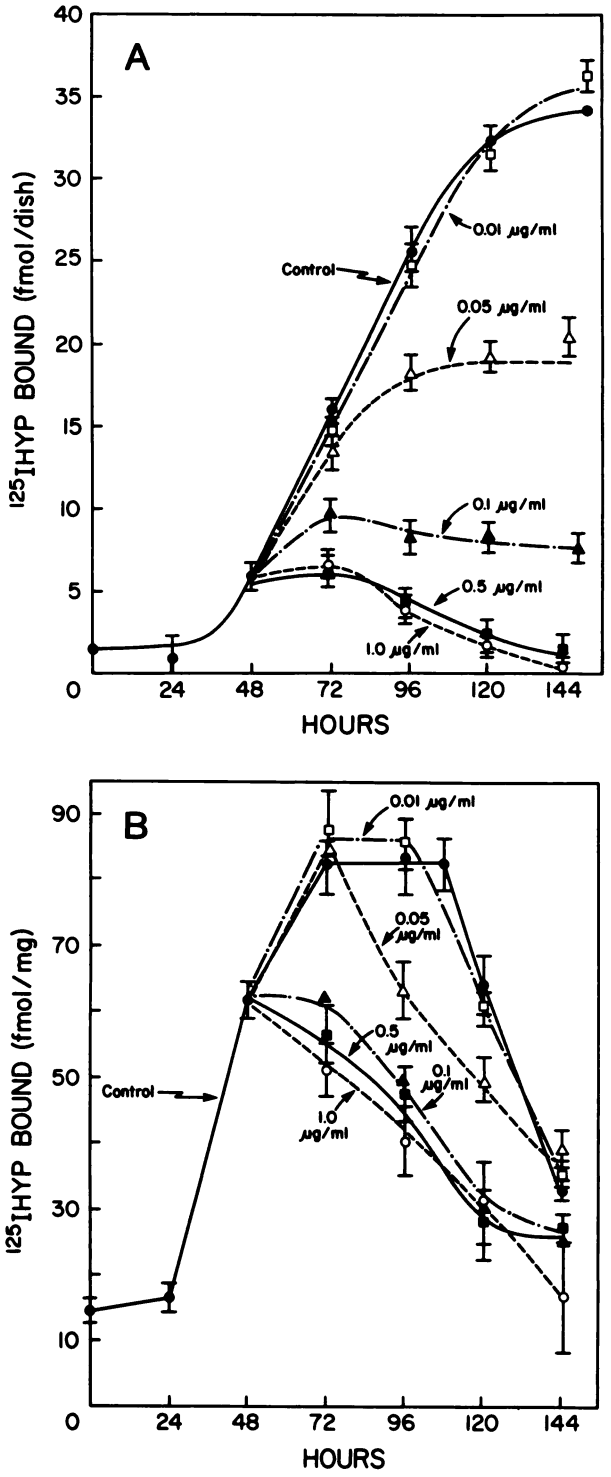

FIG. 1. *Concentration-dependent effects of tunicamycin on β-AR accumulation*

A, 8-day-old 1321N1 cell cultures were subcultured in 100-mm dishes at a density of approximately 8000 cells/cm². After 48 hr in culture, fresh DMEM containing 0, 0.01, 0.05, 0.1, 0.5, or 1.0 μg/ml of tunicamycin was added. Fresh DMEM and tunicamycin were added 48 hr after the initial drug addition. At the indicated times, the cells were lysed and the homogenates were assayed for β-AR content as described under Experimental Procedures. The data are plotted as the amount of ^{125}IHYP bound per culture dish and are the mean ± standard error of values for quadruplicate dishes for each condition. The data are representative of three similar experiments. B, the same data are plotted as receptor content per mg of protein.

number of β-AR in freshly plated cells increases from about 1000/cell to about 4000/cell. This apparent increase in the rate of β-AR synthesis occurs during the first two doublings of the cell population; thus, the cells are producing, on average, 2500 β-AR/cell/division (24 hr) over the two division cycles. After reaching an average density of 4000 β-AR per cell, the cultures produce 2000 β-AR/cell/division until confluence is reached. Thereafter, no net increase in β-AR occurs in the culture dish, and the β-AR density drops to 800–1000/cell as the cells continue to grow. Assuming reproducible plating efficiency and cell growth, the number of β-AR per culture dish represents the net receptors accumulated. Cultures of 1321N1 cells accumulate an amount of β-AR that is determined by the number of cells at confluency (100×10^6 cells/cm²) × 4000 β-AR/cell. Even though the number of cells can increase to five to six times the number of cells at confluency, the total number of β-AR (per dish) remains constant (14, 19). The cells reach confluency at 100 ± 10 hr under standard conditions of subculture and growth. The average cell-doubling time in preconfluent cultures is about 24 hr and in postconfluent culture slows to about 30–36 hr. The ratio of protein to cell (90 μg/10^6 cells) is quite constant throughout the typical 6–8-day growth period; this ratio was not altered by growth of cells in the presence of 0.05–0.25 μg/ml tunicamycin.

Since the ratio of β-AR/10^6 cells (or β-AR/mg of protein) changes significantly as a normal consequence of cell growth during the time course of most experiments, the effects of agents such as tunicamycin on β-AR expression are difficult to interpret from plots of changes in the specific activity of β-AR. Thus, in general, we have expressed results both as total β-AR (femtomoles/dish) and femtomoles of β-AR/mg of protein. For practical reasons, we have not measured cell number in all of the experiments reported here. However, the conversion of values for femtomoles of β-AR/mg of protein to values for femtomoles of β-AR/10^6 cells can be made reliably by multiplying the former values by 0.09 mg of protein/10^6 cells.

Characterization of the effects of tunicamycin on accumulation of β-AR in growing 1321N1 cells. The effect of tunicamycin on β-AR expression was examined during a typical 6-day subculture period (Fig. 1A). At the lowest concentration (0.01 μg/ml) tested, tunicamycin had no observed effect on either β-AR levels or the rate of cell growth. Thus, the triphasic expression of β-AR per cell (Fig. 1B, β-AR/mg of protein) was indistinguishable from the pattern of change observed in untreated cultures. At intermediate concentrations (0.05–0.10 μg/ml), tunicamycin caused a time-dependent inhibition of the accumulation of β-AR (Fig. 1) as well as a reduction (50–70%) in cell growth rate (Table 1). Comparison of the effects of 0.10 μg/ml tunicamycin on cell number (Table 1), total β-AR (Fig. 1A), and β-AR per cell (Fig. 1B) indicates that, during the first 24 hr of exposure of cells to 0.1 μg/ml tunicamycin, β-AR accumulation is incompletely inhibited. Thereafter, no net increase in β-AR occurs and the β-AR per cell decreases markedly by 144 hr. At higher concentrations of tunicamycin (0.5–1.0 μg/

TABLE 1

Effect of tunicamycin on cell growth

Cells were seeded and treated as described in Fig. 1, to which all time points correspond. Cells from each 100-mm dish were trypsinized and counted with an Electrozone-Celloscope (Particle Data, Inc.). The results are from one experiment. Data are presented as number of cells ($\times 10^{-6}$)/100-mm dish.

Hours in culture	Tunicamycin concentration (μg/ml)					
	0	0.01	0.05	0.1	0.5	1.0
0	0.45					
48	0.78					
72	2.07	1.62	1.60	1.57	1.18	1.27
96	3.59	3.01	2.99	1.99	1.15	1.03
120	6.69	5.22	4.45	2.44	0.88	0.64
144	10.82	9.96	6.37	3.02	0.29	0.20

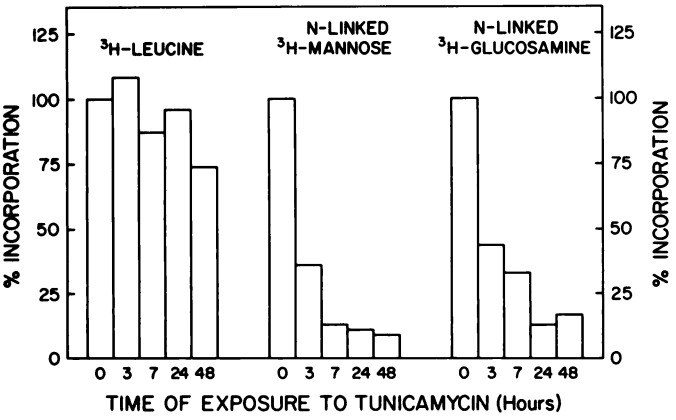

FIG. 2. *Effect of tunicamycin on protein synthesis and glycosylation*

Seven-day-old cultures were subcultured into 60-mm dishes at a density of approximately 8000 cells/cm^2. After 48 hr in culture, tunicamycin (0.1 μg/ml) was added and the incubation continued for 3, 7, 24, or 48 hr. The incorporation of [^{3}H]leucine into protein and [^{3}H]mannose and [^{3}H]glucosamine into asparagine-linked glycoproteins was determined as described under Experimental Procedures. The data are plotted as the percentage of the control incorporation of each radioactive compound per mg of protein. The data are the mean for triplicate dishes under each condition and are representative of results from three similar experiments. The amount of ^{125}IHYP bound per dish was 2.2 ± 0.2, 8.4 ± 0.2, and 2.0 ± 1.0 fmol for control cultures at 0-hr exposure to tunicamycin, control cultures after an additional 48 hr in culture, and treated cultures after 48 hr in the presence of tunicamycin, respectively.

ml), β-AR accumulation is more rapidly blocked and cell number also declines (Table 1). As a compromise between maximal effect on β-AR accumulation and minimal effect on cell growth, a concentration of 0.1 μg/ml tunicamycin was chosen for most of the experiments that follow.

The time courses of the effects of 0.1 μg/ml tunicamycin on the incorporation of [^{3}H]leucine into total protein and on incorporation of [^{3}H]mannose or [^{3}H]glucosamine into glycoprotein containing *N*-glycosidic linkages were compared (Fig. 2). It should be noted that the indicated time of exposure is the time at which a 6-hr exposure to the labeled precursor was initiated; thus, during the 7th–13th hr of exposure, tunicamycin reduced incorporation of [^{3}H]mannose into asparagine-linked glycoproteins to 10% of the incorporation observed with untreated cells. When we examined the effect of tunicamycin on incorporation of [^{3}H]mannose during a 1-hr pulse, little effect was observed prior to 4 hr (results not shown). It is clear from a number of such experiments that at least 6 hr of exposure to 0.1 μg/ml tunicamycin are required to inhibit the synthesis of glycoproteins by 70–90%.

The reversibility of the effects of tunicamycin on β-AR accumulation also was examined. Cells previously treated with 0.1 μg/ml tunicamycin for 48 hr were washed free of the drug and incubated in tunicamycin-free medium for various times (Fig. 3). Following a lag of approximately 12 hr, β-AR number recovered to control levels within 48 hr. Addition of cycloheximide (5 μg/ml) following removal of tunicamycin prevented the recovery of β-AR (Fig. 3).

As illustrated in Fig. 4, β-AR remaining after exposure of cells to tunicamycin for 24 hr exhibited the same K_d for ^{125}IPIN binding as did control β-AR. Thus, the effects of tunicamycin seem to be solely on the rate of accumulation of normal β-AR. At least if aglycoreceptors are present after tunicamycin treatment, they either do not bind the radioligand or they exhibit the antagonist-binding properties of native receptors.

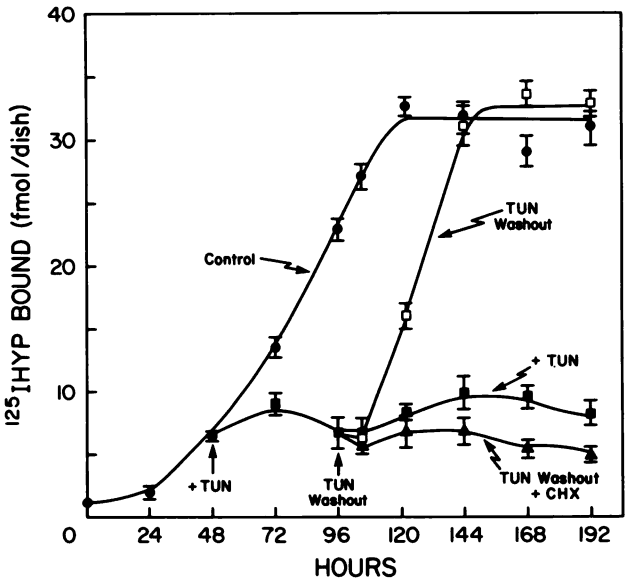

FIG. 3. *Expression of β-AR during normal growth and subsequent to incubation of 1321N1 cells with tunicamycin*

Cells were subcultured into 100-mm dishes as described under Experimental Procedures. Following 48 hr in culture, fresh DMEM was added and incubation continued in the absence (●) or presence (■) of 0.1 μg/ml tunicamycin (*TUN*). After 48 hr (i.e., 96 hr postsubculture) in the presence of tunicamycin, the cells were washed free of drug, fresh DMEM was added, and incubation was continued in the absence (□) of tunicamycin, in the continued presence of 0.1 μg/ml tunicamycin (■), or in the presence of 5 μg/ml cycloheximide (*CHX*) (▲). Fresh drug-free DMEM, DMEM plus tunicamycin, or DMEM plus cycloheximide was added to the appropriate cultures at 144 hr postsubculture. At the indicated times, the cells were lysed and β-AR content was measured in homogenates as described under Experimental Procedures. Data are the mean ± standard error of duplicate assays from three dishes for each condition. The data are representative of three similar experiments.

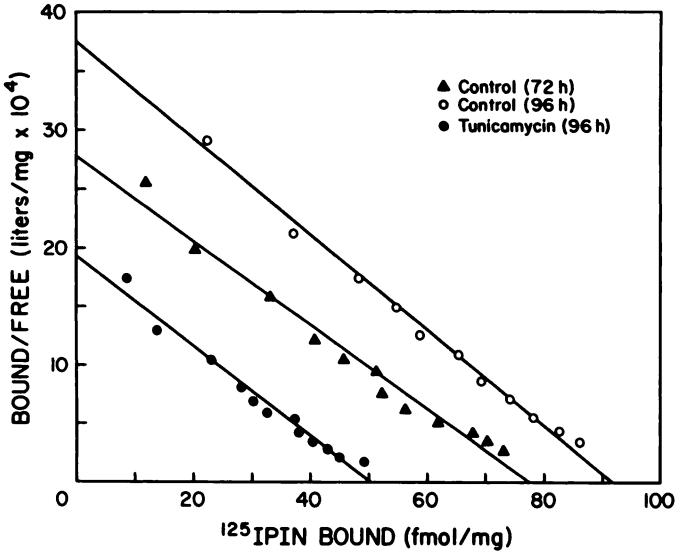

FIG. 4. *Scatchard analysis of ^{125}IPIN binding*

Cells were subcultured as described in the legend to Fig. 1. One set of cultures (▲) was hypotonically lysed and homogenates were prepared after 72 hr in culture. The medium of the remaining cultures was replenished and incubation continued for another 24 hr in the absence (○) or presence (●) of 0.1 μg/ml tunicamycin. These cultures were then hypotonically lysed and receptor content was measured in the homogenates as described under Experimental Procedures. Saturation binding isotherms were generated for each condition by varying ^{125}IPIN concentrations from 5–230 pM. The K_d values for the 72-hr control, 96-hr control, and tunicamycin-treated cells were 28, 24, and 26 pM, respectively. The B_{max} values for the 72-hr control, 96-hr control, and tunicamycin-treated cells were 78, 92, and 50 fmol/mg of protein, respectively. The data are plotted as the ratio of the amount of specifically bound ligand to free ligand (ordinate) versus the amount of specifically bound ligand (abscissa). Data points are the average of Scatchard plots from each of three culture dishes under each condition. The results are representative of two separate experiments. Lines represent the least squares fit.

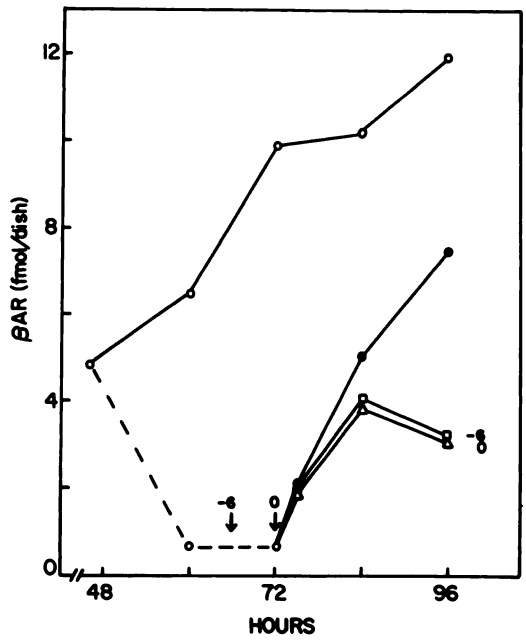

FIG. 5. *Effect of time of exposure to tunicamycin on the recovery of β-AR after down-regulation in preconfluent cultures of 1321N1 cells*

Isoproterenol (final concentration, 1.0 μM) was added to the medium of 48-hr cultures. Twelve hr after addition of the agonist, fresh medium containing 0.01 μM isoproterenol was added to the cultures and incubation continued for an additional 12 hr. Tunicamycin (0.1 μg/ml final) was added to some of the cultures at 66 (□) or 72 hr (△). At 72 hr, isoproterenol was washed from the cultures, and fresh DMEM containing no drug (●) or tunicamycin (□, △) was added. The data are the mean of triplicate determinations for each condition and are representative of two similar experiments.

Effects of tunicamycin and cycloheximide on β-AR recovery after agonist-induced down-regulation in preconfluent cultures. We have previously reported (14) that under appropriate growth conditions (i.e., in preconfluent cultures) the β-AR that are lost to detection by radioligand-binding assays following a 12-hr incubation with catecholamine can regain binding activity after removal of the catecholamine even in the presence of cycloheximide. In light of these results, it was of interest to determine the effects of tunicamycin on the recovery of "lost" receptors following exposure of 1321N1 cells to isoproterenol. Incubation of preconfluent cells for 24 hr with 0.1 μM isoproterenol resulted in a greater than 80% loss of β-AR (Figs. 5 and 6). Although tunicamycin (0.1 μg/ml) blocked β-AR accumulation during cell growth (Figs. 1 and 3), it did not block the recovery of lost β-AR after exposure of 1321N1 cells to isoproterenol (Figs. 5 and 6). That is, following removal of isoproterenol from the medium, β-AR returned to the level observed at the time of addition of isoproterenol. The initial rate of recovery in the presence of tunicamycin was similar to the rate of recovery in the absence of drug (Fig. 5). As previously reported (14), the rate of recovery in the presence of cycloheximide was considerably reduced with a $t_{1/2}$ for recovery of about 36 hr (Fig. 6B).

Since the effects of tunicamycin were apparently not maximal until after 6 hr of exposure of control cells, recovery was examined under conditions (Fig. 5) in which tunicamycin was 1) added 6 hr prior to washout of isoproterenol, or 2) added at the time of washout of isoproterenol. An amount of β-AR equivalent to the number present at the time of addition of isoproterenol was recovered even when tunicamycin was present 6 hr prior to the removal of isoproterenol.

Fig. 6 illustrates another characteristic of catecholamine-induced down-regulation of β-AR in preconfluent cultures; namely, the number of β-AR recovered in the presence of tunicamycin (Fig. 6A) or cycloheximide (Fig. 6, B and C) is related to the number of β-AR at the time of addition of isoproterenol and is independent of the duration of exposure to isoproterenol. These results are consistent with our previously stated (14) proposal that recovery of down-regulated β-AR in preconfluent cultures of 1321N1 cells does not require the synthesis of new β-AR. Of course, in cells allowed to recover in the absence of inhibitors of protein synthesis, β-AR accumulate not only as a result of the recovery process, but presumably as a result of the reinitiation of normal β-AR synthesis. In fact, as shown in Fig. 6A, the rate of accumulation of β-AR per cell upon removal of isoproterenol was equivalent to the maximal rate observed in untreated cells. Fig. 6C demonstrates that β-AR recover

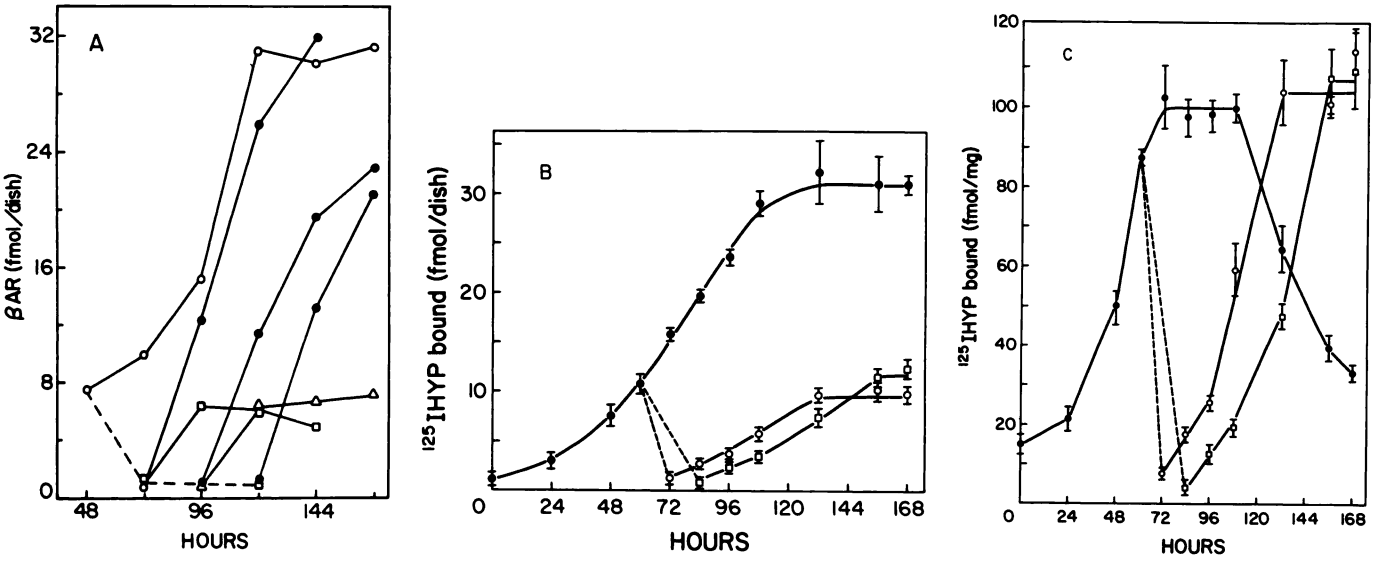

FIG. 6. *Effect of time of exposure to isoproterenol on the extent of recovery of β-AR in the presence of tunicamycin or cycloheximide*

A, at 48 hr, fresh DMEM containing isoproterenol (0.01 μM) was added to the cell cultures. Isoproterenol was washed out after 24, 48, or 72 hr of exposure and the cultures were allowed to recover in drug-free DMEM (●). Control cultures received no isoproterenol (○). Tunicamycin (0.1 μg/ml) was added to some cultures 6 hr before the removal of isoproterenol [i.e., at 66 (□) or 90 hr (△)] and maintained during the 72-hr recovery period. The data are the mean of triplicate determinations for each condition and are representative of two similar experiments. B, at 60 hr, fresh DMEM containing 0.01 μM isoproterenol was added to the cell cultures. At 72 (○) and at 84 hr (□), the cultures were washed free of isoproterenol and fresh DMEM containing 5 μg/ml cycloheximide was added. Incubation was continued until 168 hr. Under these conditions, in the presence of cycloheximide, cell growth is completely inhibited. C, the data in B are expressed as femtomoles of ^{125}IHYP/mg of protein.

in the presence of cycloheximide to the same specific activity exhibited by the control cells at the time of addition of cycloheximide to the down-regulated cells. This result is expected if all lost receptors are actually recovered since the cell number does not change during the recovery period in the presence of cycloheximide.

Effect of tunicamycin on β-AR recovery in postconfluent cultures after agonist-induced down-regulation. In contrast to the situation with preconfluent cultures, our earlier studies (14) suggested that recovery of β-AR after down-regulation in postconfluent cultures required protein synthesis. That is, whereas cycloheximide had no effect on the extent of recovery of lost β-AR in preconfluent cultures, it completely inhibited recovery in confluent cultures. The effect of tunicamycin on recovery of lost β-AR in confluent cultures of 1321N1 cells is shown in Fig. 7. In contrast to the effect of cycloheximide (14), recovery of β-AR occurred in the presence of tunicamycin. Since it was possible that 0.1 μg/ml tunicamycin would not be as effective in postconfluent as in preconfluent cultures, the effect of tunicamycin on [^{3}H]mannose incorporation into asparagine-linked total glycoprotein was examined during the recovery phase. The protocol involved a 6-hr exposure of cells to the labeled sugar during 6–12, 42–48, and 90–96 hr of recovery in the presence of 0.1 or 0.25 μg/ml tunicamycin. The per cent inhibition of sugar incorporation by tunicamycin was 60% (0.1 μg/ml, 6–12 hr), 80% (0.25 μg/ml, 6–12 hr, and 0.1 μg/ml, 42–48 hr), and greater than 90% (0.25 or 0.1 μg/ml, 90–96 hr.). Under all conditions, tunicamycin was without significant effect on β-AR recovery.

The experiments shown in Fig. 8 illustrate another approach to the study of the effect of culture density on the recovery of β-AR after down-regulation. The design of these experiments was as follows. First, down-regulation was effected in *preconfluent* cultures. Then the cultures were maintained in the continued presence of isoproterenol until a postconfluent state was reached. At this point, the isoproterenol was removed and the recovery of β-AR was examined in the presence of tunicamycin. In the experiment shown in Fig. 8A, preconfluent cultures were exposed to isoproterenol for 24 hr to cause a 90% reduction in β-AR, and β-AR were maintained at a low level by extending the exposure to isoproterenol for up to 72 hr. Recovery was initiated at 24-hr intervals by removal of isoproterenol and the incubation was continued in the presence of tunicamycin. As expected (see Figs. 5 and 6), recovery initiated at 72 and 96 hr in the presence of tunicamycin occurred only to the level of β-AR present at the beginning of down-regulation, presumably because the cultures were still in a preconfluent state. In contrast, when recovery was initiated at 120 hr, β-AR accumulated during the following 72 hr to three times the level present at the beginning of down-regulation. Thus, tunicamycin was ineffective in preventing β-AR synthesis after 160–184 hr of culture life, a time at which the cultures had achieved a confluent state. In a similar experiment (Fig. 8, B and C), preconfluent cultures were exposed to isoproterenol for 84 hr and tunicamycin was added at the time of removal of isoproterenol. Again, in contrast to the effect of preconfluent cultures, β-AR recovered in the presence of tunicamycin to a level in excess of the level present at the time of initiation of down-regulation. The difference in the rate and extent of recovery in the presence and absence of tunicamycin observed in this experiment (Fig. 8C) ap-

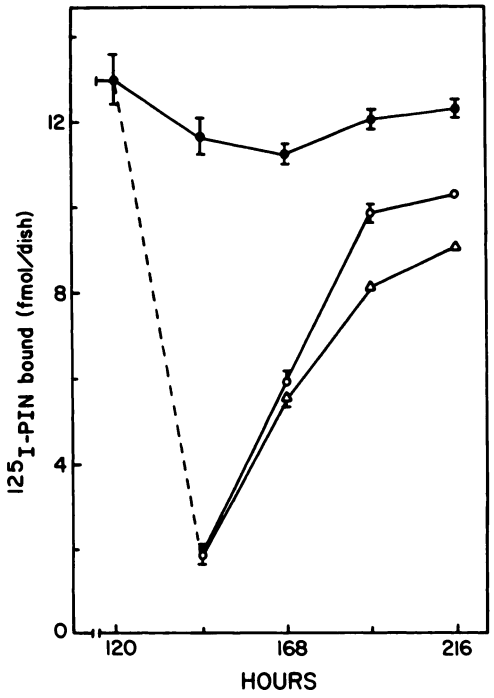

FIG. 7. *Effects of tunicamycin on the recovery of β-AR following incubation of postconfluent cultures with isoproterenol*

Cells were subcultured as described under Experimental Procedures. After 120 hr in culture, (±)-isoproterenol (final concentration, 0.1 μM) was added to the medium with fresh drug added 6 hr later. Twelve hr (132 hr postsubculture) subsequent to the addition of agonist, the dishes were washed with agonist-free DMEM, and fresh DMEM containing no additional drug (○) or containing 0.1 μg/ml tunicamycin (△) was added. Results for control cultures (●) which received no isoproterenol or tunicamycin also are presented. Receptor content was assessed at the indicated times as was described under Experimental Procedures. The data are the mean ± standard error of values from quadruplicate dishes and are representative of results of four separate experiments.

pears to be related to the number of cells in the cultures since recovery to the same specific activity (Fig. 8B) of β-AR was observed. Also, it can be noted (Fig. 8B) that cells recovering in the presence or absence of tunicamycin attained the same specific activity as control cells that were not previously exposed to isoproterenol.

DISCUSSION

Our previous studies demonstrated cell density-dependent regulation of the expression of β-AR in 1321N1 cells (14, 19). It appears that these cells have a mechanism(s) for recognizing the existence of a suboptimal level of β-AR and, under preconfluent culture conditions, will accumulate β-AR at a rate of 2000–2500 β-AR/cell/cell division time. Upon reaching a confluent state, receptor accumulation ceases and the number of receptors per cell declines directly in proportion to a continuing increase in cell number. Accordingly, we have taken into account this native regulation of receptor expression in an examination of the effects of tunicamycin on β-AR accumulation.

The marked reduction in receptor accumulation in the presence of tunicamycin, together with other indirect evidence (5–7), suggests that the β-AR is a glycoprotein the expression of which can be altered by modification of the process of dolichol pyrophosphate-mediated protein glycosylation. However, it is recognized that tunicamycin could act indirectly by influencing the synthesis of another protein involved in the cellular processing of β-AR.

Our previous experiments (14) demonstrated that β-AR accumulation in preconfluent cultures was prevented by cycloheximide and that in the presence of cycloheximide preformed receptors were not rapidly degraded ($t_{1/2}$ > 100 hr). However, such results did not unambiguously prove that the normal turnover rate of β-AR was low, since cycloheximide might have blocked the synthesis of a rapidly turning over protein that is required for receptor degradation. Reed and Lane (28) have proposed that such a protein exists on the basis of studies of insulin receptor turnover. However, the current observation that preformed receptors also are stable in the presence of tunicamycin lends support to the idea that the β-AR does not turn over rapidly in 1321N1 cells.

Lane and co-workers observed that tunicamycin blocked the accumulation of insulin receptors in 3T3 mouse fibroblasts (25, 29). Interestingly, if upon removal of tunicamycin cycloheximide was added to the cultures, active insulin receptors still accumulated. Using the heavy amino acid density shift technique (30) to unambiguously determine new receptor synthesis (25), it was shown that the primary sequence of the receptor peptide was formed during exposure to tunicamycin and prior to addition of cycloheximide. Thus, it was concluded that aglycoreceptor protein had been formed in the presence of tunicamycin and was glycosylated post-translationally to generate a functional insulin receptor. Using the same experimental protocol, we were unable to detect formation of active β-AR after the sequential exposure of 1321N1 cells to tunicamycin and cycloheximide (Fig. 3). Therefore, with results to date, we cannot differentiate between the two most likely consequences of the inhibitory effect of tunicamycin on β-AR glycosylation: 1) incomplete formation of β-AR polypeptides occurs because glycosylation is a requisite co-translational event or 2) complete translation of the polypeptide occurs but the aglycoreceptor is either rapidly degraded or is nonfunctional. It would appear that immunological techniques will be required for the further delineation of the events involved in normal β-AR synthesis.

Recovery of β-AR after down-regulation in either pre- or postconfluent cultures occurred to the level of β-AR that were present at the time of initiation of down-regulation despite the presence of tunicamycin. In preconfluent cultures, recovery occurred to the same extent in the presence of tunicamycin or cycloheximide. These results are consistent with our previous proposal (14) that β-AR lost through the process of down-regulation in preconfluent 1321N1 cells are not degraded and can be recovered without the necessity of new β-AR synthesis. To date, we have been unable to detect this lost form of the receptor except by its absence and by its capacity to recover in the presence of cycloheximide and tunicamycin.

Our previous work also had shown that even though

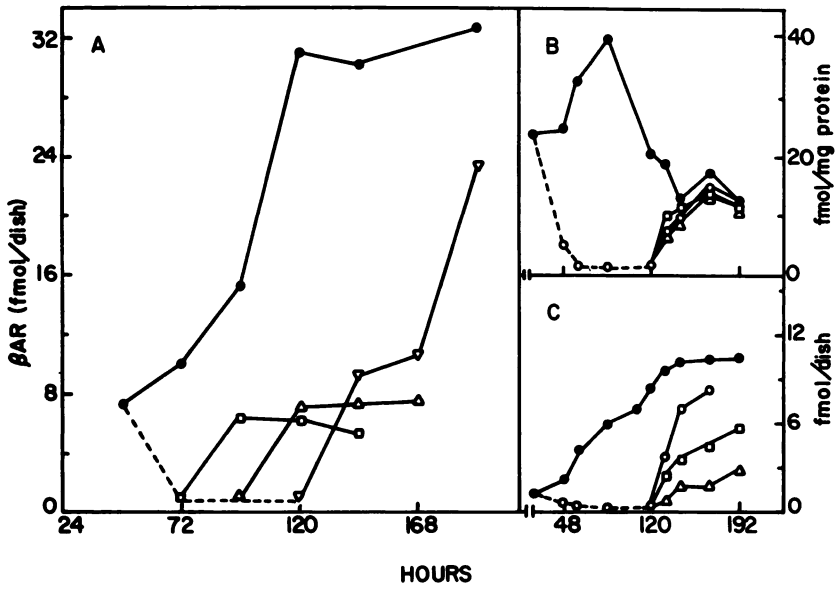

FIG. 8. *Effects of tunicamycin on recovery of β-AR after extended exposure of preconfluent cultures of 1321N1 cells to isoproterenol*

A, cultures were exposed to DMEM containing (−)isoproterenol (0.01 μM) from hour 48 to hour 120. After exposure to isoproterenol for 24 (□), 48 (△), or 72 (▽) hr, the cultures were washed free of agonist. In each case, tunicamycin (0.1 μg/ml) was added to the cultures 6 hr prior to removal of isoproterenol; incubation was continued for 72 hr in the presence of tunicamycin after removal of isoproterenol. β-AR content was measured using ^{125}I-pindolol as described in Experimental Procedures. Control cultures were maintained in drug-free DMEM (●). The data are representative of results obtained in three separate experiments. B, data from C are plotted as femtomoles of β-AR/mg of protein. C, (−)isoproterenol (0.01 μM) (○, □) or isoproterenol plus 0.1 μg/ml tunicamycin (△) was added to the cultures at 36 hr postsubculture and maintained through 120 hr. At hour 120, isoproterenol was removed and the cultures were maintained in drug-free DMEM (○) or DMEM containing 0.1 μg/ml tunicamycin (□, △).

cycloheximide did not block recovery of down-regulated β-AR in preconfluent cells it completely blocked recovery in postconfluent cells; thus, we proposed (14) that recovery in postconfluent cultures required β-AR synthesis or the synthesis of a protein involved in β-AR expression. The observation (Fig. 7) that tunicamycin did not block recovery in postconfluent cells was surprising and favored the latter conclusion, with the provision that the "other" protein was not an asparagine-linked glycoprotein. Nonetheless, in experiments in which β-AR synthesis was measured directly by an increase in mass due to incorporation of heavy (^{2}H, ^{13}C, ^{15}N) amino acids, we have shown that recovery of down-regulated receptors in postconfluent 1321N1 cell cultures involves β-AR synthesis per se (21). One is left with the seemingly anomalous results that whereas tunicamycin appeared to block β-AR synthesis in preconfluent cells it did not block β-AR synthesis during recovery from down-regulation.

In this study, we have examined the effects of tunicamycin on β-AR accumulation in four different experimental circumstances: 1) during normal growth in preconfluent cultures; 2) during recovery in preconfluent cultures after down-regulation in preconfluent cultures; 3) during recovery after down-regulation in postconfluent cultures; 4) during recovery in postconfluent cultures after down-regulation in preconfluent cultures. In the first circumstance, tunicamycin prevents β-AR accumulation probably by blockade of co- or post-translational glycosylation. In the second circumstance tunicamycin apparently blocks the synthesis of new β-AR, but does not prevent the reappearance of β-AR equivalent to the number present at the time of down-regulation. Thus, in preconfluent cultures, tunicamycin discriminates between the processes of normal synthesis and recovery. Since cycloheximide exhibits the same pattern of discrimination (14), we conclude that recovery of β-AR lost by down-regulation in preconfluent cultures does not require protein synthesis.

The situation in postconfluent cells is clearly different. First, it is probable that under normal growth conditions β-AR are synthesized slowly, if at all, in postconfluent cells. Certainly, no net accumulation of β-AR occurs in such cultures (14, 19). Second, if both down-regulation and recovery procedures are carried out with postconfluent cells, recovery of β-AR occurs via synthesis of new receptors (21).

A more complicated protocol involved down-regulation of β-AR in preconfluent cultures, followed by continued growth of the cells in the presence of isoproterenol until confluent cultures were obtained. Recovery of β-AR in these cultures upon removal of isoproterenol occurred in the presence of tunicamycin to an extent much in excess of the level of receptors present at the time of down-regulation. Thus, new β-AR must have been synthesized. It is interesting that the number of β-AR accumulating under these conditions did not exceed the number expected in cultures of 1321N1 cells growing under normal conditions. In other words, the specific activity (β-AR/mg of protein) achieved in the recovery phase was the same as that of naive cultures (see Fig. 8B). Thus, although β-AR synthesis can take place in postconfluent 1321N1 cells under these special conditions, the extent

of β-AR synthesis appears to be governed by a cell density-dependent mechanism similar to that operative in naive cells (19).

Our studies of the effects of tunicamycin and cycloheximide (14) during growth of 1321N1 cells together with direct studies (21) of β-AR synthesis in this cell line lead to the following conclusions. 1) The fate of isoproterenol-induced down-regulated β-AR is different depending on culture conditions. 2) In preconfluent cultures, the apparently lost β-AR can be recovered without the involvement of protein synthesis. Preconfluent cells exposed to isoproterenol for up to 48 hr are capable of recovery of lost β-AR in the presence of cycloheximide or tunicamycin to the level present prior to down-regulation. Thus, it would appear that such lost β-AR are actually sequestered within the cell in a form that does not bind radioligands but that can be recovered. We have no information on the localization of β-AR during exposure of preconfluent cells to isoproterenol. 3) In postconfluent cultures, exposure to isoproterenol apparently results in degradation of β-AR. The β-AR that recover upon removal of isoproterenol are newly synthesized (21) and such recovery is blocked by cycloheximide. 4) In preconfluent, growing 1321N1 cells, tunicamycin blocks the incorporation of [^{3}H]mannose into asparagine-linked glycoproteins and blocks the accumulation of β-AR. However, in postconfluent cultures undergoing β-AR synthesis during recovery from β-AR down-regulation, tunicamycin has little effect on receptor accumulation even though it blocks [^{3}H]mannose incorporation by 60–90%. Such results suggest that the modes of β-AR synthesis in the two states (confluent versus preconfluent) are different. Growing cells accumulate β-AR at about 2500 molecules/cell/24 hr, whereas β-AR are recovered in postconfluent cells (±tunicamycin) at a rate of about 500 molecules/cell/24 hr. This reduced rate of synthesis of β-AR and the lack of sensitivity of the process to tunicamycin clearly define two differences in the modes of synthesis of β-AR in pre- and postconfluent cells.

One can speculate that upon attainment of confluency the normal process of β-AR synthesis and/or post-translational processing is markedly changed. The loss of sensitivity to tunicamycin in postconfluent cultures must be, at least in part, a β-AR-selective modification since the cells continue to exhibit tunicamycin-sensitive general glycoprotein synthesis. We have not established that the marked decline in β-AR per cell during normal cell growth after confluence is directly related to the change in sensitivity of β-AR synthesis to tunicamycin.

We have considered the possibility that β-AR are synthesized as aglycoproteins in postconfluent cultures of 1321N1 cells. Even if the normal rate of translation was maintained in this situation, the rate of appearance of measurable β-AR might be markedly reduced if the polysaccharide portion of the molecule was important for β-AR stability or for the efficiency of its transport to the plasma membrane. In this regard, it has been shown that treatment of plasma membrane preparations with endoglycosidase F leads to a reduction in the apparent molecular weight of β-AR (5). Nonetheless, it has been reported in a preliminary communication that the modified β-AR exhibited normal ligand-binding characteristics and were able to reconstitute isoproterenol-sensitive adenylate cyclase activity in β-AR-replete vesicles (31). In preliminary studies, we have shown that the β-AR recovered after down-regulation in postconfluent cultures of 1321N1 cells exhibit normal affinity for radioligands (antagonists), mediate activation of adenylate cyclase, and undergo down-regulation upon exposure to isoproterenol. Studies are currently underway to determine, by more direct analysis, if aglyco-β-AR are formed in postconfluent cultures of 1321N1 cells.

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REFERENCES

1. Ross, E. M., and A. G. Gilman. Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.* **49**:533–564 (1980).
2. Limbird, L. E. Activation and attenuation of adenylate cyclase. *Biochem. J.* **195**:1–13 (1981).
3. Lefkowitz, R. J., J. M. Stadel, and M. G. Caron. Adenylate cyclase-coupled beta adrenergic receptors: structure and mechanisms of activation and desensitization. *Annu. Rev. Biochem.* **52**:159–186 (1983).
4. Rothman, J. E., and J. Lenard. Membrane asymmetry. *Science* **195**:743–749 (1977).
5. Stiles, G. L., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Mammalian β-adrenergic receptors: distinct glycoprotein populations containing high mannose or complex type carbohydrate chains. *J. Biol. Chem.* **259**:8655–8663 (1984).
6. Stadel, J. M., R. G. L. Shorr, L. E. Limbird, and R. J. Lefkowitz. Evidence that a β-adrenergic receptor associated guanine nucleotide regulatory protein conveys guanosine 5′-*O*-(3-thiotriphosphate)-regulatory adenylase cyclase activity. *J. Biol. Chem.* **256**:8718–8723 (1981).
7. Meier, K. E., D. R. Sterfeld, and P. A. Insel. How different are alpha- and beta-adrenergic receptors? *Fed. Proc.* **42**:691 (1983).
8. Lefkowitz, R. J., M. R. Wessels, and J. M. Stadel. Hormones, receptors, and cyclic AMP: their role in target cell refractoriness. *Curr. Top. Cell. Regul.* **17**:205–230 (1980).
9. Harden, T. K. Agonist-induced densensitization of the β-adrenergic receptor-linked adenylate cyclase. *Pharmacol. Rev.* **35**:5–32 (1983).
10. Harden, T. K., Y. F. Su, and J. P. Perkins. Catecholamine-induced desensitization involves an uncoupling of beta-adrenergic receptors and adenylate cyclase. *J. Cyclic Nucleotide Res.* **5**:99–106 (1979).
11. Su, Y. F., T. K. Harden, and J. P. Perkins. Catecholamine-specific desensitization of adenylate cyclase: evidence for a multistep process. *J. Biol. Chem.* **255**:7410–7419 (1980).
12. Wessels, M. R., D. Mullikin, and R. J. Lefkowitz. Selective alteration in high affinity agonist binding: a mechaniam of beta-adrenergic receptor desensitization. *Mol. Pharmacol.* **16**:10–20 (1979).
13. Green, D. A., and R. B. Clark. Adenylate cyclase coupling proteins are not essential for agonist-specific desensitization of lymphoma cells. *J. Biol. Chem.* **256**:2105–2108 (1981).
14. Doss, R. C., J. P. Perkins, and T. K. Harden. Recovery of β-adrenergic receptors following long-term exposure of astrocytoma cells to catecholamine: role of protein synthesis. *J. Biol. Chem.* **256**:12281–12286 (1981).
15. Homburger, V., M. Lucas, B. Cantau, J. Barabe, J. Penit, and J. Bockaert. Further evidence that desensitization of β-adrenergic sensitive adenylate cyclase proceeds in two steps: modification of the coupling and loss of β-adrenergic receptors. *J. Biol. Chem.* **255**:10436–10444 (1980).
16. Mukherjee, C., M. G. Caron, and R. J. Lefkowitz. Catecholamine-induced subsensitivity of adenylate cyclase associated with loss of β-adrenergic receptor binding sites. *Proc. Natl. Acad. Sci. USA* **72**:1945–1949 (1975).
17. Shear, M., P. A. Insel, K. L. Melmon, and P. Coffino. Agonist-specific refractoriness induced by isoproterenol. *J. Biol. Chem.* **251**:7572–7576 (1976).
18. Terasaki, W. L., G. Brooker, J. de Vellis, D. Inglish, C. Y. Hsu, and R. D. Moylan. Involvement of cyclic AMP and protein synthesis in catecholamine refractoriness. *Adv. Cyclic Nucleotide Res.* **9**:33–52 (1978).
19. Harden, T. K., S. J. Foster, and J. P. Perkins. Differential expression of components of the adenylate cyclase system during growth of astrocytoma cells in culture. *J. Biol. Chem.* **254**:4416–4422 (1979).
20. Hubbard, S. C., and R. J. Ivatt. Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **50**:555–583 (1981).
21. Waldo, G. L., R. C. Doss, J. P. Perkins, and T. K. Harden. Use of a density-

shift method to assess β-adrenergic receptor synthesis during recovery from catecholamine-induced down-regulation. *Mol. Pharmacol.* **26**:424–429 (1984).
22. Harden, T. K., B. B. Wolfe, and P. B. Molinoff. Binding of iodinated beta-adrenergic antagonists to proteins derived from rat heart. *Mol. Pharmacol.* **12**:1–15 (1976).
23. Witkin, K. M., and T. K. Harden. A sensitive equilibrium binding assay for soluble β-adrenergic receptors. *J. Cyclic Nucleotide Res.* **7**:235–246 (1981).
24. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
25. Ronnett, G. V., and M. D. Lane. Post-translational glycosylation-induced activation of aglycoinsulin receptor accumulation during tunicamycin treatment. *J. Biol. Chem.* **256**:4704–4707 (1981).
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
27. Keenan, R. W., R. L. Hamill, J. L. Occolowitz, and A. D. Elbein. Biological activities of isolated tunicamycin and streptovirudin fractions. *Biochemistry* **20**:2969–2973 (1981).
28. Reed, R. C., and M. D. Lane. Insulin receptor synthesis and turnover in differentiating 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci. USA* **77**:285–289 (1980).
29. Reed, B. C., G. V. Ronnett, and M. D. Lane. Role of glycosylation and protein synthesis in insulin receptor metaboliam by 3T3-L1 mouse adipocytes. *Proc. Natl. Acad. Sci. USA* **78**:2908–2912 (1981).
30. Hu, A. S. L., R. M. Bock, and H. O. Halvorson. Separation of labelled from unlabelled proteins by equilibrium density gradient sedimentation. *Anal. Biochem.* **4**:489–504 (1962).
31. Benovic, J. L., R. A. Cerione, J. Codina, L. Birnbaumer, R. J. Lefkowitz, and M. G. Caron. Deglycosylation of the pure β-adrenergic receptor. *Fed. Proc.* **43**:1579 (1984)
32. Doss, R. C., T. K. Harden, and J. P. Perkins. Role of protein glycosylation in the synthetic processing of β-adrenergic receptors. *Fed. Proc.* **41**:1534 (1982).
33. Kramarcy, N. R., T. K. Harden, and J. P. Perkins. Cell density-dependent effects of tunicamycin on β-adrenergic receptor expression in astrocytoma cells. *Fed. Proc.* **43**:874 (1984).

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