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Turnover of β_1- and β_2-Adrenergic Receptors after Down-Regulation or Irreversible Blockade

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SUMMARY

The turnover of β_1- and β_2-adrenergic receptors was measured after both isoproterenol-induced down-regulation and irreversible blockade of receptors. Changes in the density of receptors were quantified using the radioligands ^{125}I-iodopindolol and ^{125}I-iodocyanopindolol. Treatment of intact L6 myoblasts or C_6 glioma cells with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) inactivated β-adrenergic receptors on membranes prepared from these cells. At a concentration of 100 μM EEDQ, more than 90% of β_1- and β_2-adrenergic receptors were inactivated within 2 hr of treatment. Recovery of β-adrenergic receptors on intact cells after inactivation by EEDQ required more than 24 hr and was prevented by cycloheximide, an inhibitor of protein synthesis. The kinetics of recovery of the density of receptors were analyzed in terms of a model that allows estimation of the rate constants for receptor appearance in and disappearance from the membrane, assuming that the rate of appearance of receptors is constant and the rate of disappearance of receptors is proportional to the number of receptors. β_2-Adrenergic receptors on L6 myoblasts were incorporated into the membrane at a rate of 28 fmol/mg of protein/hr and had a half-life of 12.6 hr. On C_6 glioma cells, β_1- and β_2-adrenergic receptors appeared at rates of 13.3 and 6.6 fmol/mg of protein/hr, respectively, with half-lives of 9.4 and 6.4 hr. Recovery of receptors on C_6 cells after isoproterenol-induced down-regulation was inhibited by cycloheximide. The rate of recovery of β_1- and β_2-adrenergic receptors was reduced after treatment with isoproterenol for 8 hr when compared to recovery after treatment with EEDQ. The major effect of treatment with isoproterenol was a persistent decrease in the rate of appearance of β_1- and β_2-adrenergic receptors (rate of synthesis and insertion into the membrane after treatment with isoproterenol = 4.0 fmol/mg of protein/hr). Since treatment with isoproterenol did not alter the rate of cell division or total protein synthesis, the isoproterenol-induced alteration was probably a specific effect on the rate of synthesis of β-adrenergic receptors.

On the basis of the rank order of potency of catecholamines for stimulation of β-adrenergic receptor-mediated responses in different tissues, β-adrenergic receptors were divided into two classes (1). β_1- and β_2-adrenergic receptors coexist in most tissues and are sometimes present on the same cell (2). Although they are pharmacologically distinct, β_1- and β_2-adrenergic receptors that coexist in a tissue or cell frequently appear to be functionally similar. For example, both subtypes are present on subclones of C_6 glioma cells and both mediate activation of adenylate cyclase (2). In dog atrium, both β_1- and β_2-adrenergic receptors are coupled to adenylate cyclase (3) and both mediate isoproterenol-induced shortening of the action potential duration (4).

Differences between β_1- and β_2-adrenergic receptors are observed when either turnover or agonist-induced down-regulation of β-adrenergic receptors is studied. In rat kidney, the half-life of receptors during agonist-induced down-regulation is similar for the two subtypes, but during recovery from down-regulation, the half-life of β_1-adrenergic receptors is much longer than that of β_2-adrenergic receptors (5). Treatment of rats with the atypical agonist pindolol selectively down-regulates β_2-adrenergic receptors in heart and lung (6). Similarly, exposure of C_6 glioma cells to pindolol or celiprolol causes a large decrease in the density of β_2-adrenergic receptors with little or no change in the density of β_1-adrenergic receptors (7). Thus, when extent of down-regulation of receptors is used as a measure of intrinsic activity, pindolol and celiprolol appear to be much more efficacious at β_2- than at β_1-adrenergic receptors, although neither drug activates adenylate cyclase. These phenomena could be explained if β_2-adrenergic receptors are coupled more efficiently than β_1-adrenergic receptors to an effector mechanism that does not involve synthesis of cyclic AMP, and this effector or some other mechanism regulates the degradation or synthesis of β_2-adrenergic receptors independently of that of β_1-adrenergic receptors.

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ABBREVIATIONS: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazine-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; IPIN, iodopindolol; ICYP, iodocyanopindolol; *r*, rate constant for synthesis and insertion of receptors into the membrane; *k*, rate constant for degradation of receptors.

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Several methods are available for measuring turnover of receptors. One approach is to determine the appearance or disappearance of receptors labeled with heavy amino acids (8). The rate of appearance of a product of degradation of receptors can sometimes be measured. For example, when acetylcholine receptors on skeletal muscle are labeled with ^{125}I-α-bungarotoxin, the release of specifically bound ligand from the muscle appears to be a measure of the rate of degradation of the receptor (9). Rate constants for degradation and synthesis of receptors can also be calculated by determining the kinetics of change from one steady state to another (5) or the kinetics of recovery of the density of receptors after down-regulation or irreversible blockade (10). It is assumed that the kinetics of recovery can be described by a model in which the steps involved in synthesis of receptors and insertion into the membrane, described by the rate constant, r, occur at a constant rate. The steps involved in degradation of the receptors, described by the rate constant, k, are assumed to take place at a rate that is proportional to the density of receptors. These assumptions have been independently validated for several types of receptors (8, 11, 12). Data for recovery of receptors in several systems are well described by this model (5, 10, 13), but the procedure provides valid estimates of basal rate constants only if synthesis or degradation of receptors is not altered by the agonist or irreversible antagonist used to alter the steady state density of receptors. A number of compounds have been developed as irreversible ligands for β-adrenergic receptors. To inactivate receptors to study coupling to second messenger systems or the kinetics of repopulation, halogenated derivatives of antagonists have been most useful (13–16).

EEDQ is a peptide bond-forming reagent that has been used to irreversibly inactivate α-adrenergic and dopamine receptors (17–19). EEDQ is thought to alkylate the carboxyl group of an acylamino acid, forming a mixed carbonic anhydride and free quinoline (20). The mixed carbonic anhydride reacts with a sterically accessible nucleophilic group to inactivate the receptor.

We now report that EEDQ irreversibly inactivates β_1- and β_2-adrenergic receptors on cultured cells. When the recovery of receptors was studied after treatment with EEDQ, the degradation rate constants for β_1- and β_2-adrenergic receptors were similar. The difference in steady state densities of the subtypes was due to a difference in the rate constant, r. Recovery of the density of receptors occurred more slowly after isoproterenol-induced down-regulation than after treatment with EEDQ, indicating that treatment with isoproterenol caused an alteration in turnover of receptors that persisted after removal of the agonist.

Methods

Tissue culture. The L6 skeletal muscle cell line was developed by Yaffe (21) from 3-methylcholanthrene-treated primary cultures of newborn rat thigh muscle. A nonfusing variant of this cell line was used for these studies between passage 3 and 20 after cloning. The C_6 glioma cell line was developed by Benda *et al.* (22) from rat glial tumors induced by injections of *N*-nitrosomethylurea. C_6 cells of the BU1 subclone between passage 57 and 70 were used. Both cell lines were grown in DMEM (Flow Laboratories, Inc., McLean, VA) supplemented with 5% (C_6 cells) or 10% (L6 cells) fetal bovine serum (Sterile Systems, Inc., Logan, UT) in an atmosphere of 10% CO_2/90% air at 37°. L6 cells were plated at 16,000 cells/cm^2 in 100-mm-diameter dishes (Falcon; VWR Scientific, San Francisco, CA), subcultured on day 2, or fed on day 3 and harvested on day 5. C_6 glioma cells were plated at 20,000 cells/cm^2 in 150-mm-diameter dishes (Lux; Miles Scientific, Naperville, IL), fed or subcultured on day 3, and harvested on day 5. Cells were lysed by replacing the growth medium with ice-cold hypotonic buffer (1 mM Na^+-HEPES, pH 7.4, 2 mM EDTA). After swelling for 10–15 min, the cells were removed with a rubber policeman and centrifuged at 24,000 × g for 20 min. The resulting crude membrane fraction was resuspended with a Brinkmann Polytron homogenizer at setting 6 for 10 sec in Tris-isosaline (20 mM Tris-HCl, pH 7.4, and 0.9% NaCl) and stored at −70°.

Drug treatment. All drugs were freshly prepared and sterilized by filtration before being added to the growth medium. EEDQ was dissolved in methanol to a concentration of 50 mM, then diluted as required with water and growth medium. Isoproterenol and sotalol were dissolved in DMEM, and cycloheximide was dissolved in water at 1000 times the desired final concentration. To assess the effect of drug treatments on the growth of cells, the number of cells excluding trypan blue dye was determined with a hemacytometer immediately and 24 hr after treatment. Protein synthesis was measured by growing cells for 4 hr in methionine-free DMEM supplemented with ^{35}S-methionine (4 μCi/ml). The cells were washed with phosphate-buffered saline and dissolved in 0.3 N NaOH. Protein was precipitated in 10% trichloroacetic acid and redissolved in NaOH for liquid scintillation spectroscopy and protein determination by the method of Lowry *et al.* (23).

Receptor binding assay. Before use, the membrane preparation was thawed, incubated for 10 min at 37° to release residual drug from the membranes, centrifuged at 24,000 × g for 20 min, and resuspended in Tris-isosaline. Aliquots of the membrane preparation were added to assay tubes containing 20 mM Tris-HCl (pH 7.4), 0.9% NaCl, 1.1 mM ascorbic acid, 0.0004% bovine serum albumin, 100 μM guanosine 5′-triphosphate, radioligand, and appropriate drugs. (−)-Isoproterenol (200 μM) was used to define nonspecific binding. The total amount of radioligand bound was less than 10% of its free concentration. ^{125}I-IPIN (2.2 Ci/μmol) and ^{125}I-ICYP (2.2 Ci/μmol) were prepared and purified by modifications of the method of Barovsky and Brooker (24) as described previously (25). Incubations were carried out at 37° for 24 min (^{125}I-IPIN) or 70 min (^{125}I-ICYP) and were stopped by the addition of 10 ml of 0.9% NaCl in 10 mM Tris-HCl (25°, pH 7.4) to each assay. The samples were filtered through glass-fiber filters (Schleicher and Schuell No. 30) and washed with an additional 10 ml of buffer. The radioactivity retained on the filter was counted in an LKB 1274 or a Beckman 4000 gamma counter at an efficiency of 75%. Protein was determined according to the method of Bradford (26) using bovine serum albumin as the standard.

Because C_6 cells have both β_1- and β_2-adrenergic receptors (2, 7), the results of binding assays are more accurate when ^{125}I-ICYP is used, since ^{125}I-ICYP is less selective than ^{125}I-IPIN for β_2-adrenergic receptors, and can be used in competition experiments at a concentration that is high relative to its K_D value (25). The density of receptors was assessed on membranes prepared from C_6 cells in triplicate in an assay volume of 1.0 ml using ^{125}I-ICYP at concentrations from 3 to 55 pM. The relative proportion of β_1- and β_2-adrenergic receptors was determined by inhibition of the binding of 55 pM ^{125}I-ICYP with 21 concentrations of the β_1-selective drug ICI 89,406. Since L6 cells have only β_2-adrenergic receptors, binding assays were carried out using ^{125}I-IPIN, which approaches equilibrium rapidly. The density of receptors on membranes prepared from these cells was determined by Scatchard analysis of the binding of various concentrations of ^{125}I-IPIN from 10 to 200 pM. Assays were performed in duplicate in a volume of 0.25 ml.

Analysis of data. Inhibition curves were analyzed by nonlinear regression analysis using NEWFITSITES2 on the National Institutes of Health-supported PROPHET computer system. The data were fit to the following two-site equation:

$$B = \frac{B_1 \cdot IC_1}{I + IC_1} + \frac{B_2 \cdot IC_2}{I + IC_2}$$

where B is the amount of radioligand bound, B_1 and B_2 are the total

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number of sites of each subtype labeled by this concentration of radioligand, I is the concentration of the competing ligand, and IC_1 and IC_2 are the concentrations of competing ligand that inhibit 50% of the binding to each subtype (27). To determine the density of β_1- and β_2-adrenergic receptors in a membrane preparation, the relative percentage of each subtype was multiplied by the B_{max} value derived from Scatchard analysis of a simultaneously performed saturation isotherm. Data for recovery of receptors were fit by nonlinear regression to a steady state equation (Eq. 1, described in Results) with the PROPHET mathematical modeling program, MLAB, using a Marquardt-Levenberg method. All statistical comparisons were made using the Newman-Keuls multiple range test except where noted. Where it is stated that no significant difference was found, $p > 0.05$.

Materials. C_6 cells were obtained from Dr. Mark Dibner (E. I. Du Pont de Nemours and Co., Wilmington, DE). (−)-Pindolol, (−)-cyanopindolol (Sandoz, Hanover, NJ), ICI 89,406 (ICI America, Inc., Wilmington, DE), and sotalol (Mead Johnson and Co., Evansville, IN) were gifts. (−)-Isoproterenol bitartrate, guanosine 5′-triphosphate, EEDQ, and cycloheximide were obtained from Sigma Chemical Co. (St. Louis, MO). Na^{125}I (2.2 Ci/μmol) and ^{35}S-methionine (1.13 Ci/μmol) were purchased from New England Nuclear (Boston, MA).

Results

Effect of treatment with EEDQ or isoproterenol on C_6 cells. Treatment with EEDQ or isoproterenol did not significantly alter the number of viable C_6 cells (Table 1). Cell growth, determined by counting the number of viable cells 24 hr after treatment, was also unchanged. Incorporation of ^{35}S-methionine into total cellular protein was not changed either 0–4 or 20–24 hr after treatment with EEDQ or isoproterenol (Table 1). After exposure to cycloheximide (5 μg/ml of DMEM) for 8 hr, more than 90% of L6 or C_6 cells were viable (data not shown).

Effect of EEDQ on the density of β-adrenergic receptors. Treatment of L6 myoblasts with EEDQ resulted in a concentration- and time-dependent loss of β_2-adrenergic receptors (Fig. 1A). The loss of receptors was almost complete after treatment with 100 μM EEDQ for 1 hr (mean density ± SEM = 9 ± 1% of control) with only a small additional decrease after 2 hr (6 ± 1% of control). The decrease in binding of ^{125}I-IPIN was characterized by a decrease in the B_{max} with no consistent change in the affinity of the receptors for ^{125}I-IPIN (Fig. 1B).

TABLE 1

Effect of treatment of C_6 glioma cells with EEDQ or isoproterenol on growth rate and protein synthesis

Each value represents the mean ± standard error of three experiments in which the number of viable cells or incorporation of ^{35}S-methionine was determined. C_6 cells were grown in 60-mm-diameter dishes, for determination of protein synthesis, or 150-mm-diameter dishes as described in Methods. Cells were treated with 100 μM EEDQ for 2 hr or 5 μM isoproterenol (ISO) for 8 hr. Viability of cells was determined immediately after treatment by counting in a hemacytometer the number of cells capable of excluding trypan blue. Cell division was evaluated using the same test 24 hr after treatment. Treatments were scheduled so that cells from each group and control cells were harvested simultaneously. The same schedule was used to assess protein synthesis except that cells were incubated with ^{35}S-methionine for 4 hr either 0–4 or 20–24 hr after the termination of drug treatment, then harvested as described in Methods. Results are expressed as number of viable cells/dish and cpm of ^{35}S-methionine/mg of protein.

Treatment	Time after treatment	Viable cells	^{35}S-Methionine incorporated
	hr		
Vehicle		93 ± 15 × 10^6	1.04 ± 0.12 × 10^5
EEDQ	0	93 ± 20 × 10^6	1.02 ± 0.13 × 10^5
EEDQ	24	87 ± 14 × 10^6	1.01 ± 0.11 × 10^5
ISO	0	96 ± 14 × 10^6	1.01 ± 0.13 × 10^5
ISO	24	81 ± 12 × 10^6	1.09 ± 0.16 × 10^5

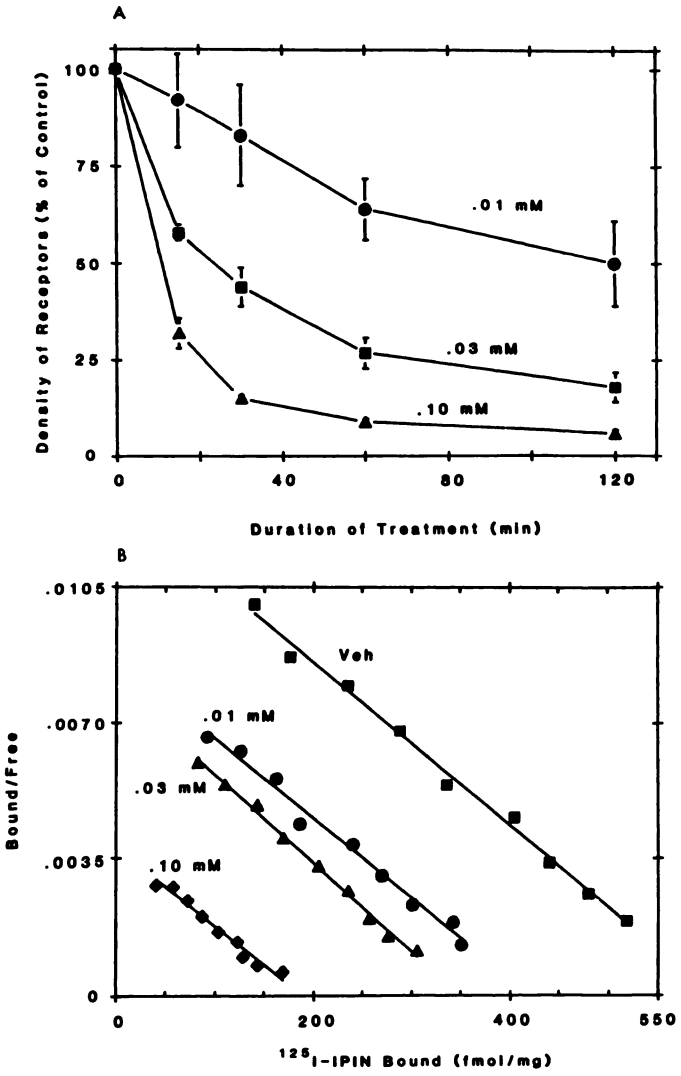

Fig. 1. Effect of treatment with EEDQ on β-adrenergic receptors on L6 cells. L6 cells were treated with EEDQ, and the membranes were prepared as described in Methods. The density of receptors was determined by Scatchard analysis of binding isotherms for ^{125}I-IPIN. A. Each *point* is the mean ± standard error of B_{max} values for the binding of ^{125}I-IPIN determined in three independent experiments. The data are plotted as percentage of control versus the duration of treatment with the indicated concentration of EEDQ. The control density of receptors was 565 ± 45 fmol/mg of protein. B. Scatchard plots from a representative experiment. Cells were treated for 15 min with the indicated concentrations of EEDQ or vehicle (*Veh*). Each *point* is the average of duplicate assays. The amount of radioligand bound per mg of protein divided by the free concentration of radioligand is plotted against the amount of radioligand bound per mg of protein. The free concentration was determined by subtracting the amount of radioligand bound from the added concentration of radioligand.

According to the Newman-Keuls multiple range test, only the pK_D for cells treated with 100 μM EEDQ for 60 min (10.15 ± 0.03, n = 3) was significantly different from control (mean control pK_D ± SE = 10.32 ± 0.06; q-crit = 4.65, $p < 0.05$).

The density of β-adrenergic receptors on membranes prepared from C_6 glioma cells was also decreased by EEDQ, reflecting a decrease in the density of both β_1- and β_2-adrenergic receptors (Fig. 2). In the presence of 100 μM EEDQ, the time course of the loss of β_2-adrenergic receptors on C_6 cells was almost identical to that of β_2-adrenergic receptors on L6 cells. Although EEDQ appeared to be slightly less potent at β_1-

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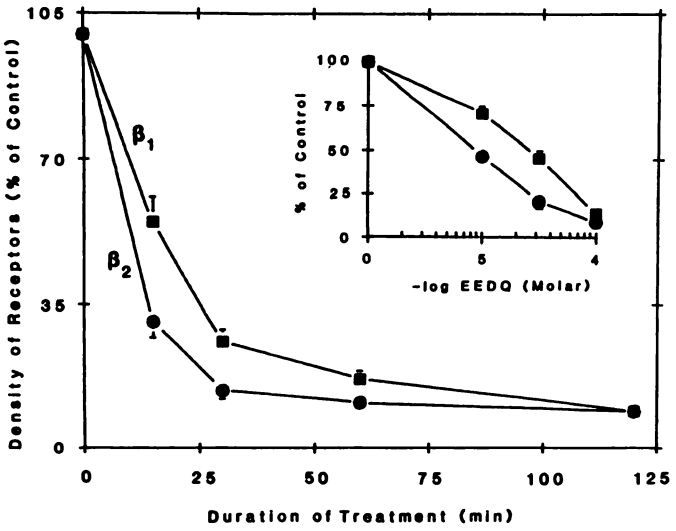

Fig. 2. Effect of treatment with EEDQ on β-adrenergic receptors on C_6 glioma cells. The density of β_1- or β_2-adrenergic receptors, expressed as percentage of control, is plotted against the duration of incubation of C_6 cells with 100 μM EEDQ. Membranes were prepared as described in Methods, and the density of each subtype was determined by multiplying the proportion of receptors represented by that subtype by the total number of receptors calculated from Scatchard analysis, as described in Methods and Fig. 1. Each *point* represents the mean ± standard error of three independent experiments with assays carried out in triplicate. The control density of β_1-adrenergic receptors was 92 ± 25 fmol/mg of protein and the control density of β_2-adrenergic receptors was 62 ± 10 fmol/mg of protein. *Inset:* The density of β_1 (■)- or β_2 (●)-adrenergic receptors is plotted versus the concentration of EEDQ with which the cells were treated for 1 hr. The control densities of β_1- and β_2-adrenergic receptors were 84 ± 4 fmol/mg of protein and 70 ± 3 fmol/mg of protein, respectively.

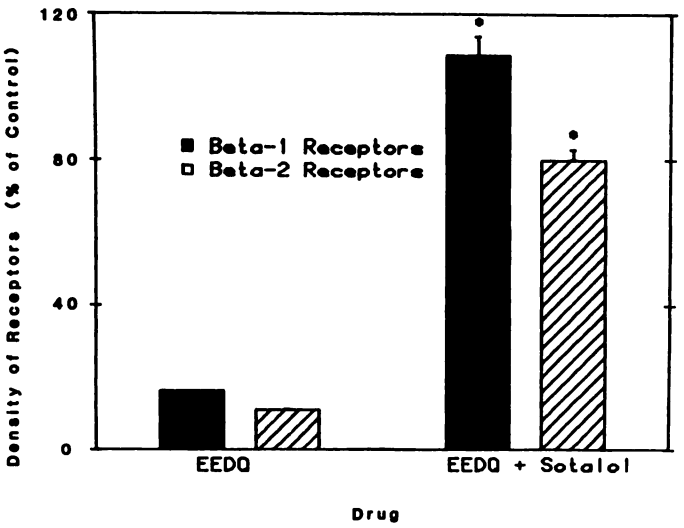

Fig. 3. Protection by sotalol from inactivation by EEDQ. The densities of β_1- and β_2-adrenergic receptors, expressed as percentage of control, are shown after treatment with EEDQ in the absence or presence of sotalol. Cells were treated with 50 μM sotalol or vehicle and, 5 min later, 100 μM EEDQ was added for 1 hr. The densities of β_1- and β_2-adrenergic receptors before exposure to EEDQ were 58 ± 8 and 57 ± 5 fmol/mg of protein, respectively. *, $p < 0.01$ compared to EEDQ without sotalol, as determined by a *t* test for paired means.

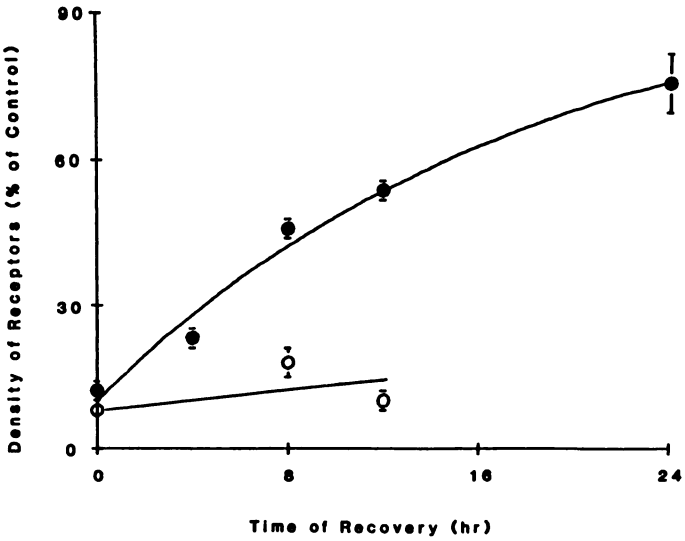

Fig. 4. Repopulation of β-adrenergic receptors on L6 myoblasts. The values shown are the mean ± standard error of three experiments. L6 cells were treated with 100 μM EEDQ for 1 hr, then rinsed three times for 1 min each with drug-free medium, and the time course of recovery in drug-free medium was determined (●). The treatments were scheduled so that all cells were harvested simultaneously. The *curve* represents the best fit of the data to Eq. 1. Values for the rate constants *r* and *k*, derived from the modeling procedure, are given in Table 2. Some cells (○) were maintained in medium containing cycloheximide (5 μg/ml of DMEM) after removal of EEDQ. The results are plotted as percentage of control density of receptors against the time of recovery. The density of receptors on control cells was 513 ± 49 fmol/mg of protein.

adrenergic receptors (Fig. 2, *inset*), after treatment with 100 μM EEDQ for 2 hr the density of each subtype was reduced by more than 90%. The affinity of β-adrenergic receptors on C_6 cells for ^{125}I-ICYP was not significantly altered by treatment with EEDQ (control $pK_D \pm SE = 11.14 \pm 0.13$, $n = 3$).

In other experiments, the ability of the hydrophilic antagonist sotalol to protect β-adrenergic receptors from inactivation by EEDQ was determined. Treatment of C_6 cells with 100 μM EEDQ decreased the density of β_1- and β_2-adrenergic receptors to 17 ± 1% and 10 ± 1% of control, respectively. The density of β_1-adrenergic receptors after treatment with EEDQ in the presence of 50 μM sotalol was not significantly different from control (mean ± SE = 109 ± 5%, $n = 3$), whereas the density of β_2-adrenergic receptors was 80 ± 3% of control (Fig. 3).

Recovery of receptors after inactivation by EEDQ. L6 myoblasts were treated with 100 μM EEDQ for 1 hr. The medium containing EEDQ was removed and replaced with drug-free DMEM, and the cells were allowed to recover for up to 24 hr. Repopulation of β-adrenergic receptors on these cells occurred slowly, so that by 24 hr after removal of EEDQ the density of receptors on membranes prepared from the cells was only 76 ± 6% of the density of receptors on control cells (Fig. 4). Reappearance of receptors was prevented by cycloheximide (5 μg/ml of DMEM). The K_D value for the binding of ^{125}I-IPIN did not change during the period of recovery (data not shown). The rate of recovery of the binding of ^{125}I-IPIN was not altered by the presence of 50 μM sotalol in the growth medium (data not shown).

Recovery of receptors on C_6 cells after treatment with 100 μM EEDQ for 2 hr took place in DMEM containing 50 μM sotalol, to prevent re-binding of residual EEDQ. The density of receptors recovered at a rate similar to that for receptors on L6 myoblasts. After 24 hr in DMEM containing 50 μM sotalol and no EEDQ, the density of β_1-adrenergic receptors increased from 12 to 87% of control and the density of β_2-adrenergic receptors increased from 10 to 86% of control (Fig. 5). The

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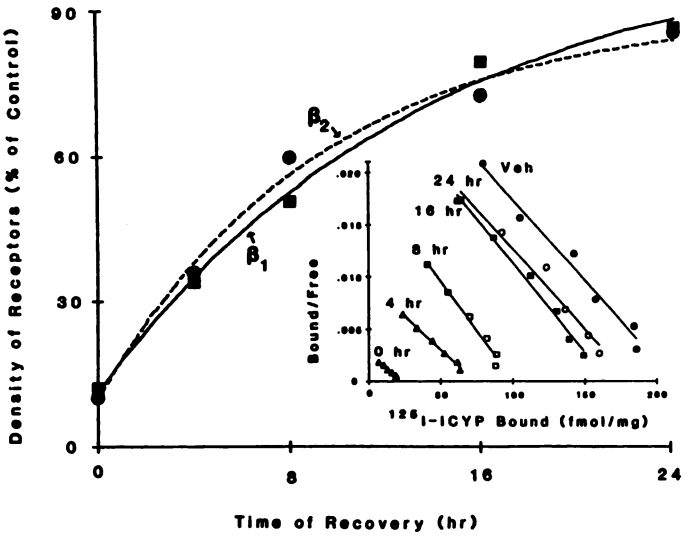

Fig. 5. Repopulation of β-adrenergic receptors on C_6 cells after inactivation of EEDQ. The mean of three experiments is shown for the time course of recovery of β_1- and β_2-adrenergic receptors after treatment with 100 μM EEDQ for 2 hr. The *curve* represents the best fit of the data to Eq. 1 for β_1 (■)- and β_2 (●)-adrenergic receptors. Values for the rate constants *r* and *k* are given in Table 2. After treatment, cells were rinsed for 1 min with medium, then rinsed again for 15 min. At that time medium that contained 50 μM sotalol was added to the cells. Cells in the control group were grown in the presence of sotalol for 24 hr prior to harvesting. The treatments were scheduled in such a way that all cells in an experiment were harvested simultaneously. Membranes were prepared as described in Methods except that the last step, a 10-min incubation in drug-free medium and recentrifugation, was repeated. The density of β_1- and β_2-adrenergic receptors was determined as described in Methods. *Inset:* Scatchard plots from a representative experiment are shown. Triplicate assays were performed at each point, and the results were analyzed as described in Fig. 1. The duration of incubation in drug-free medium after treatment with EEDQ is shown for each *line*.

affinity of β-adrenergic receptors for ^{125}I-ICYP throughout the period of recovery (Fig. 5, *inset*) was not significantly different from control (mean control $pK_D \pm SE = 11.17 \pm 0.07$). Cycloheximide (5 μg/ml of medium) inhibited the reappearance of receptors; after recovery for 12 hr in the presence of cycloheximide, neither the density of β_1-adrenergic receptors (16 ± 2% of control) nor that of β_2-adrenergic receptors (8 ± 2% of control) was significantly different from that seen immediately after treatment with EEDQ.

Determination of rate constants for synthesis and degradation of receptors. The steady state density of receptors is a function of the rate of synthesis or appearance and the rate of degradation or disappearance of receptors. Acetylcholine (8, 11), insulin (12), and α- (10, 13), and β-adrenergic (5) receptors appear to fit a model in which the rate of synthesis of receptors is constant, and the rate of degradation is proportional to the concentration of receptors. According to this model the rate of repopulation of receptors after inactivation can be described by the equation:

$$R_t = \frac{r}{k}(1 - e^{-kt}) + R_o e^{-kt} \quad (1)$$

where R_t is the density of receptors at time *t*, *r* is the rate of synthesis and insertion into the membrane, *k* is the rate constant for degradation, and R_o is the density of receptors at time 0. An attractive feature of this model is that no assumption is made about the steady state density of receptors after drug treatment, and the analysis yields a prediction (r/k) for the eventual steady state density after complete recovery.

The repopulation of β-adrenergic receptors on L6 cells was fit to Eq. 1 (Fig. 4), yielding estimates for the constants *r* and *k* of 28 fmol/mg of protein/hr and 0.055/hr, respectively, with a half-life ($t_{1/2}$) for the receptors of 12.6 hr. For all fits to Eq. 1 in this study, the values for these parameters were virtually identical whether R_o was constrained to the experimentally determined values for R_o or determined by the fitting procedure. The rates of recovery of both β_1- and β_2-adrenergic receptors on C_6 cells after treatment with EEDQ were also fit to Eq. 1 (Fig. 5). β_1-Adrenergic receptors were incorporated into the membrane at a rate of 7.7% of control/hr, with a $t_{1/2}$ for degradation of 9.4 hr, whereas β_2-adrenergic receptors appeared at a rate of 9.8% of control/hr with a $t_{1/2}$ of 6.4 hr (Table 2). The control density of β_1-receptors was much greater than the control density of β_2-receptors, yielding values for the rate of synthesis (*r*) of 13.3 and 6.6 fmol/mg of protein/hr for β_1- and β_2-adrenergic receptors, respectively.

Repopulation of β_1- and β_2-adrenergic receptors after down-regulation. C_6 glioma cells were treated with isoproterenol, then washed and allowed to recover in medium that contained sotalol, to prevent re-binding of residual isoproterenol. Recovery of both β_1- and β_2-adrenergic receptors occurred more slowly than after inactivation of the receptors of EEDQ ($p < 0.001$ for the effect of drug, that is, isoproterenol versus EEDQ, according to a two-way analysis of variance). The effect of isoproterenol was greater on repopulation of β_1- than β_2-adrenergic receptors ($F = 190.9$ and 24.0, respectively, compared to recovery after treatment with EEDQ). After 24 hr, the density of β_1-adrenergic receptors recovered to only 37% of control (Fig. 6A). According to the ratio r/k (Table 2), if the recovery of the density of receptors continued at the same rate as observed during the first 24 hr after down-regulation, a new steady state density of receptors of only 50% of the control density would be reached. If the period of recovery were extended, the density of receptors would possibly recover to the control density as the effect of isoproterenol faded. The decrease in the ratio r/k during the first 24 hr of recovery, however, indicates that either the rate constant for synthesis was decreased or that for degradation was increased. In these experiments the decreased rate of recovery was primarily due to a decrease in the rate of synthesis of receptors from 13.3 fmol/mg of protein/hr to 4.0 fmol/mg of protein/hr. The density of β_2-adrenergic receptors increased from 12 to 69% of the

TABLE 2

Computer-assisted modeling of the kinetics of repopulation

The observed data for repopulation of receptors after treatment with EEDQ or isoproterenol (ISO) were fit to Eq. 1, yielding the values given ± the standard deviation of regression. The steady state density of receptors predicted by Eq. 1 is equal to the ratio r/k, where *r* is the rate constant of synthesis and *k* the rate constant for degradation of receptors. Values for *r* are expressed as per cent of control density of receptors/hr. The control density was 513 ± 49 fmol/mg of protein for β_2-receptors on L6 cells, 172 ± 13 fmol/mg of protein for β_1-receptors, and 68 ± 6 fmol/mg of protein for β_2-receptors on C_6 cells. Units for *k* and the half-life of the receptors ($t_{1/2}$) are hr^{-1} and hr, respectively.

Subtype/cell	Drug	r	k	$t_{1/2}$
β_2/L_6	EEDQ	5.55 ± 1.31	0.055 ± 0.025	12.6
β_1/C_6	EEDQ	7.72 ± 1.16	0.073 ± 0.018	9.4
β_2/C_6	EEDQ	9.77 ± 1.46	0.108 ± 0.022	6.4
β_1/C_6	ISO	2.29 ± 1.38	0.045 ± 0.059	15.4
β_2/C_6	ISO	5.69 ± 1.03	0.076 ± 0.020	9.1

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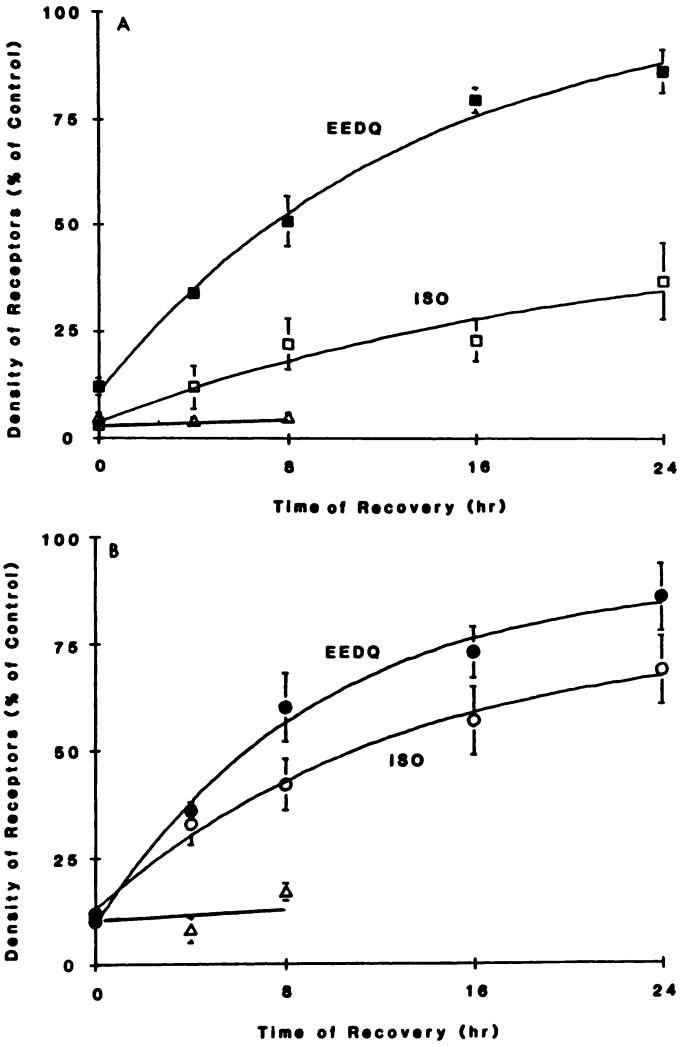

Fig. 6. Comparison of repopulation of β-adrenergic receptors after treatment with EEDQ or isoproterenol. Data are shown for repopulation of β_1 (A)- and β_2 (B)-adrenergic receptors after drug treatments. Each *point* represents the mean ± standard error of three experiments in which cells were treated with 100 μM EEDQ (■, ●) or 5 μM isoproterenol (*ISO*, □, ○). The *solid lines* represent the best fit of the data to Eq. 1. Values for the rate constants *r* and *k* are given in Table 2. In some experiments (△) cells were grown in cycloheximide for 8 hr after treatment with isoproterenol. The data for cells treated with EEDQ are the same as in Fig. 5. In each experiment, one group of cells was treated with EEDQ and another group was treated in parallel with isoproterenol. The drug treatment, rinsing, and recovery were performed as described in the legend to Fig. 5, except that some cells were treated with 5 μM isoproterenol for 8 hr, and isoproterenol was re-added after the initial 4 hr. Recovery of receptors was in the presence of 50 μM sotalol, and cells in the control groups were grown in sotalol for 24 hr. According to a 2-factorial blocked analysis of variance, the rate of recovery of either β_1- or β_2-adrenergic receptors following exposure to isoproterenol was significantly different from that following exposure to EEDQ ($p < 0.001$).

control density of receptors within 24 hr after treatment with isoproterenol (Fig. 6B). The data for recovery of β_2-adrenergic receptors were fit well by Eq. 1, but the values for the rate constants *r* and *k* yielded by Eq. 1 predicted that a new steady state density of 78% of the control density of receptors would be reached. This was mainly the result of a decrease in the rate constant *r* (4.0 fmol/mg of protein/hr). The $t_{1/2}$ of β_2-adrenergic receptors was increased slightly after down-regulation (9.1 hr) compared to that after inactivation of receptors (Table 2).

The affinity of β-adrenergic receptors for ^{125}I-ICYP was not altered following exposure to isoproterenol (mean control pK_D ± SE = 11.07 ± 0.07). Recovery of both β_1- and β_2-adrenergic receptors was prevented by cycloheximide (Fig. 6, A and B). In other experiments, C_6 cells were treated with isoproterenol to determine whether short-term desensitization with isoproterenol was sufficient to decrease the rate of repopulation of receptors. After 30 min, the isoproterenol was replaced by 100 μM EEDQ for 2 hr. The density of receptors determined 24 hr after treatment was the same as the density of receptors 24 hr after treatment with EEDQ alone (data not shown.)

Discussion

Neither growth rate nor total protein synthesis was altered by treatment with isoproterenol or EEDQ as used in this study. The number of viable cells, assessed by ability to exclude trypan blue dye, was not significantly different from the number of vehicle-treated cells immediately or 24 hr after exposure to EEDQ or isoproterenol, and incorporation of ^{35}S-methionine into trichloroacetic acid-precipitated protein was not significantly changed. The effects of treatment with EEDQ and isoproterenol on β-adrenergic receptors were apparently specific alterations in the density and regulation of β-adrenergic receptors, rather than nonspecific effects related to toxicity or inhibition of total protein synthesis.

EEDQ is an alkylating agent that inactivates α_1- and α_2-adrenergic receptors (17, 28) as well as D_1 and D_2 dopamine receptors (18, 19, 28). The present results show that EEDQ also inactivates both β_1- and β_2-adrenergic receptors on cultured cells. At a concentration of 100 μM, treatment with EEDQ caused a nearly complete loss of the binding of radioligands to both subtypes of receptors on membranes prepared from the cells. The inactivation was probably irreversible, since the density of receptors was decreased with no change in the affinity of the receptors for the radioligand, and recovery of binding on intact cells required more than 24 hr. Recovery was blocked by treating the cells with cycloheximide. The presence of the antagonist sotalol prevented inactivation of the receptors by EEDQ, indicating that EEDQ acts at or near the ligand-binding site.

EEDQ causes little inactivation of β-adrenergic receptors *in vivo*[1] (28). At the concentrations of EEDQ needed to be effective *in vivo*, the mortality rate for rats would be prohibitively high. However, EEDQ is commercially available, and, because unbound drug can easily be reduced below levels that affect β-adrenergic receptors, EEDQ is well suited for some types of experiments. For example, an irreversible antagonist can be used to analyze the interactions of an agonist with receptors by the method of Furchgott (29), in which dose response curves for an agonist-stimulated response are compared before and after irreversible inactivation of a fraction of the receptors. As stated by Furchgott (29), conclusions based on this method about the potency of agonists and the percentage of functional receptors will be invalid if residual unbound antagonist is present after treatment to cause an apparently competitive inhibition of stimulation by an agonist. Repopulation of receptors after treatment with an irreversible antagonist will not reflect basal rates of appearance and disappearance if unbound antagonist is present at a concentration that will inactivate

[1] K. A. Neve and P. B. Molinoff, unpublished observations.

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additional receptors during the period of recovery. These problems are easier to prevent when using a compound such as EEDQ that has a relatively low potency at β-adrenergic receptors.

After treatment of cells with EEDQ, β-adrenergic receptors on C_6 and L6 cells recovered to approximately 80% of control density of receptors within 24 hr. One or both of two patterns of recovery would be characteristic of an effect of EEDQ on the rate of turnover of receptors. First, if either the rate constant for synthesis or that for degradation were changed, the receptors would be likely to recover to a new steady state density, since the steady state density (R_{ss}) is a function of the rates of synthesis and degradation ($R_{ss} = r/k$). Second, if r or k were initially changed but returned to control during the period of recovery, the rate of repopulation would not be fit well by Eq. 1. The observed data for β_2-adrenergic receptors on L6 cells and β_1- and β_2-adrenergic receptors on C_6 cells were fit well by the model described in Eq. 1, which assumes that incorporation of receptors into the membrane is linear with time and disappearance from the membrane and degradation is proportional to the number of receptors, and the computer fit predicted that the density of receptors would recover to approximately the control density of receptors. Thus, the computer-derived values for r and k probably represent approximations of the rates for synthesis and degradation of receptors that are basal rates under these culture conditions. The rate constant for synthesis, r, probably describes a multi-step process that includes the synthesis and insertion of receptors into the membrane. The rate constant for degradation, k, probably decribes multiple steps including the internalization and degradation of receptors.

Rates of turnover of β_1- and β_2-adrenergic receptors *in vivo* have been compared (5, 16), but the subtypes were on different cells or in separate tissues, so that differences between the subtypes could be attributed to nonspecific differences in metabolism or protein synthesis. In the present study, the rate constants for degradation of the two subtypes on C_6 cells were similar, as indicated by the results of the computer-assisted analysis (Table 2) and the similar time courses of recovery of receptors after inactivation by EEDQ, when the densities of β_1- and β_2-adrenergic receptors were expressed as a percentage of the control densities of β_1- and β_2-adrenergic receptors (Fig. 5). This suggests that the difference between the steady state densities of β_1- and β_2-adrenergic receptors on these cells (172 ± 13 and 68 ± 6 fmol/mg of protein, respectively) is due to the greater rate of synthesis of β_1-adrenergic receptors (13.3 fmol/mg of protein/hr) as compared to that of β_2-adrenergic receptors (6.6 fmol/mg of protein/hr).

Recovery of both β_1- and β_2-adrenergic receptors occurred more slowly after down-regulation by isoproterenol than after inactivation by EEDQ. Recovery of binding was probably not due to recycling of receptors, since recovery was prevented by cycloheximide. Although the half-life of each subtype was increased, the major effect of treatment with isoproterenol appeared to be a decrease in the rate of appearance of β_1-adrenergic receptors fro 13.3 to 4.0 fmol/mg of protein/hr and the rate of appearance of β_2-adrenergic receptors from 6.6 to 4.0 fmol/mg of protein/hr. Scarpace *et al.* (30) reported that the presence of an antagonist accelerates the recovery of β-adrenergic receptors after down-regulation, suggesting that residual agonist, or catecholamine in the serum, reduces the rate of recovery from down-regulation. Since recovery in the present experiments took place in the presence of the antagonist sotalol, it is unlikely that residual isoproterenol accounts for the decreased rate of recovery of receptors on cells treated with isoproterenol compared to cells exposed to EEDQ. Although exposure to isoproterenol for 8 hr decreased the rate of recovery of receptors, exposure to isoproterenol for 30 min, which does not cause a large decrease in the density of receptors, was not sufficient to reduce the rate of recovery following subsequent treatment with EEDQ.

Several lines of evidence indicate that the observed persistent alteration in the recovery of receptors after treatment with isoproterenol was a specific decrease in the rate of turnover of receptors rather than a nonspecific decrease in total protein synthesis. 1) The decrease in the rate of recovery of β_1-adrenergic receptors was greater than that of β_2-adrenergic receptors. A nonspecific alteration in protein synthesis would be expected to affect both subtypes to the same extent. 2) The rate of cell division was not changed after treatment with isoproterenol. 3) Neither treatment with EEDQ nor that with isoproterenol altered the incorporation of ^{35}S-methionine into total cellular protein, indicating that total protein synthesis was not altered.

The regulation of β-adrenergic receptors apparently differs according to the cell line being studied or the growth phase of the cell line. For example, Sladeczek *et al.* (13) used C_6 cells 10–20 days after subculturing, at which time cell division has almost stopped. Under these conditions, synthesis of receptors is entirely dependent on cell division; the number of receptors per cell does not recover after treatment with an irreversible antagonist (13). After down-regulation the number of receptors per cell does recover, but recovery is not prevented by cycloheximide. In contrast, in 1321N1 astrocytoma cells, the number of receptors per cell increases in preconfluent cultures and decreases after confluency has been achieved. Recovery from isoproterenol-induced down-regulation is prevented by cycloheximide in confluent but not in preconfluent cultures (31).

The present experiments were carried out using C_6 cells during exponential growth, and the density of β-adrenergic receptors was regulated in a manner different from that previously reported for 1321N1 cells or C_6 cells after cell division has stopped. During the growth phase, the number of receptors per cell and per mg of protein was constant during the interval after inoculation (3.5–5 days) used for drug treatments, but the density of receptors per dish increased as the number of cells increased.[2] The number of receptors per cell recovered after either inactivation or down-regulation, and recovery was prevented by cycloheximide. The turnover of β-adrenergic receptors measured in the present study was faster than previously reported *in vivo* or *in vitro* (5, 15, 25). Because C_6 cells during exponential growth were used, it seems reasonable to assume that the turnover of receptors was nonspecifically accelerated by high rates of protein synthesis during cell division. Thus, it has been speculated that the rate of turnover of β-adrenergic receptors is so low that molecular probes for mRNA synthesis may not be useful because the signal is undetectable (13), but C_6 cells in exponential growth may provide a system in which effects of experimental manipulations of mRNA synthesis can be directly measured when probes become available.

[2] K. A. Neve and P. B. Molinoff, unpublished observations.

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These results suggest that agonist-induced down-regulation of β-adrenergic receptors causes a specific alteration in the rate of appearance of receptors that persists after the agonist is removed. If down-regulation or recovery from down-regulation is used to assess the turnover of receptors, it should not be assumed that the results reflect basal rates of turnover. Doss *et al.* (32) reported that the appearance of β-adrenergic receptors in preconfluent cultures of 1321N1 cells, when the number of receptors per cell is increasing, is prevented by tunicamycin, an inhibitor of glycosylation, but that recovery from down-regulation in confluent cultures is not prevented by tunicamycin. The present observation of a decreased rate of appearance of receptors after down-regulation suggests that, rather than a difference between preconfluent and confluent cultures, the selective effects of tunicamycin may reflect differences between the properties of β-adrenergic receptors that appear during receptor turnover under basal conditions or after down-regulation. Experiments are under way to investigate this possibility and to compare functional characteristics of β-adrenergic receptors under these conditions.

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