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Desensitization of Catecholamine-Stimulated Adenylate Cyclase and Down-Regulation of *Beta*-Adrenergic Receptors in Rat Glioma C6 Cells

Role of Cyclic AMP and Protein Synthesis

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SUMMARY

When exposed to the *beta*-agonist (−)-isoproterenol, rat glioma C6 cells exhibited a time- and concentration-dependent reduction in isoproterenol responsiveness (desensitization) and a loss of *beta*-adrenergic receptors (down-regulation). Other agents, such as dibutyryl cyclic AMP, isobutylmethylxanthine, and cholera toxin, all of which elevate intracellular cyclic AMP levels, also induced receptor down-regulation but at a much slower rate than isoproterenol. Loss of *beta*-receptors was detected with intact cells, cell lysates, and cell membranes. Receptor loss was accompanied by a reduction in isoproterenol-stimulated cyclic AMP production and adenylate cyclase activity. For a given amount of receptor loss, this reduction was much greater with isoproterenol than with other agents. In addition, the concentration of isoproterenol required for half-maximal stimulation of cyclic AMP production was increased in cells treated with isoproterenol but not with isobutylmethylxanthine or dibutyryl cyclic AMP. The affinity of *beta*-receptors for the agonist was also lower in membranes from cells treated with isoproterenol but not the other agents. Prior treatment of the cells with cycloheximide inhibited receptor loss by isoproterenol but did not prevent desensitization or reduced affinity of *beta*-receptors for the agonist. Cycloheximide also blocked the loss of receptors induced by dibutyryl cyclic AMP and, in addition, prevented a reduction in agonist-stimulated adenylate cyclase activity. We propose that desensitization is mediated in rat glioma C6 cells only by agonists and is not dependent on either cyclic AMP or protein synthesis. Down-regulation can be induced both by agonists and by cyclic AMP and does depend on protein synthesis. Thus, desensitization and down-regulation can occur independently.

INTRODUCTION

Beta-adrenergic agonists mediate their effects on target cells by binding to specific surface receptors and stimulating adenylate cyclase. Prolonged exposure of cells to the agonist causes cells to become refractory to further stimulation by the agonist. This process of desensitization sometimes is accompanied by a loss of *beta*-adrenergic receptors (down-regulation) and may be homologous or heterologous (1–13). It is generally accepted that agonist-mediated homologous desensitization involves an "uncoupling" of the *beta*-receptor from the regulatory component of adenylate cyclase, which remains responsive to other effectors of the enzyme (3–10). In heterologous desensitization, the cyclase is less responsive to other effectors (10–13).

It has been proposed that cyclic AMP and protein synthesis are involved in the refractory state induced in rat glioma C6-2B cells (14–16). Thus, cells exposed to dibutyryl cyclic AMP, IBMX[2] or cholera toxin became refractory to ISO and ISO-treated cells became refractory to the toxin. Although similar results were observed in rat glioma C6 cells by Koschel (17), others have demonstrated that agonist-mediated desensitization in rat glioma C6 cells does not involve cyclic AMP or protein synthesis, is homologous and appears to involve uncoupling of the *beta*-receptors from the regulatory component (5, 6). Recently, Moylan *et al.* reported that cyclic AMP caused a loss of *beta*-receptors in rat glioma C6 cells (18). In this present paper, we have further

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[2] The abbreviations used are: IBMX, 3-isobutyl-1-methylxanthine; ISO, isoproterenol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DHA, dihydroalprenolol; [^{125}I]IPIN, (−)-[^{125}I]iodopindolol.

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examined the role of cyclic AMP and protein synthesis in desensitization and down-regulation in rat glioma C6 cells (19). We also have explored whether these two phenomena can be separated.

Experimental Procedures

Materials. ISO, IBMX, (±)-propranolol, and N^6, $O^{2'}$-dibutyryl cyclic AMP were obtained from Sigma Chemical Company (St. Louis, Mo.). Cholera toxin was from Calbiochem-Behring (La Jolla, Calif.). (−)-Pindolol was a generous gift from Dr. D. Hauser (Sandoz, Basel, Switzerland) and was iodinated as described by Barovsky and Brooker (20). (−)-[*propyl*-1,2,3-^{3}H]Dihydroalprenolol (90 Ci/mmol), (−)-[^{125}I]iodocyanopindolol, (±)-[^{125}I]iodocyanopindolol, and (±)-[^{125}I]iodohydroxybenzylpindolol (all 2200 Ci/mmol) were obtained from New England Nuclear Corporation (Boston, Mass.). [α-^{32}P]ATP (25–30 Ci/mmol) was from ICN (Irvine, Calif.). All other chemicals were of analytical grade.

Cell culture. Rat glioma C6 cells obtained from American Type Culture Collection were cultured as described previously and used between passages 40 and 52 (21). For most experiments, the maintenance subcultures were grown in medium supplemented with 2% fetal calf serum in order to delay the transition that these cells undergo with continuous passage (6, 21). In order to induce desensitization and down-regulation, the cells were incubated with various effectors in serum-free medium for different times. The cells then were washed twice with ice-cold phosphate-buffered saline (pH 7.4). Cells to be used for cyclic AMP accumulation studies were incubated 10 min at 4° with a buffered salts solution (20).

Preparation of lysates and membranes. Lysates were prepared by washing the cells once more with 5mM Hepes/1 mM $MgSO_4$ (pH 8.0) and keeping them in the latter buffer on ice for 10 min. The cells then were detached by scraping and vigorous pipetting and vortexed to lyse them. Membranes were prepared from the cells essentially as described previously (6, 10). The cells, washed once more and suspended in 2 mM Tris-HCl (pH 7.4)/1 mM EDTA/0.2 mM dithiothreitol, were disrupted with a Brinkmann Polytron and centrifuged at 600× *g* for 12 min. The supernatant then was centrifuged at 36,000× *g* for 30 min. The membranes were suspended in 10 mM Tris-HCl (pH 7.7)/1 mM $MgCl_2$/0.1 mM EDTA and assayed the same day or suspended in 250 mM sucrose/10 mM Tris-HCl (pH 7.6)/5 mM $MgCl_2$/1 mM dithiothreitol and frozen in liquid nitrogen for later assays.

Binding to beta-receptors. Binding of [^{125}I]IPIN to intact cells grown in multicluster trays (6 × 35 mm) was determined as described previously (20), except the cells were incubated with 200 pM [^{125}I]IPIN at 4° for 2 hr. Binding of labeled antagonist to lysates and membranes was determined by established methods (6, 10). Briefly stated, lysates or membranes (30–60) μg of protein) were incubated with the labeled antagonist in 50 mM Tris-HCl (pH 7.7)/1 mM $MgCl_2$/0.1 mM EDTA at 30° for 30–90 min. The incubation mixtures were filtered under vacuum on Whatman GF/B glass-fiber filters with five washes of 4 ml of 10 mM Tris-HCl (pH 7.7)/1 mM $MgCl_2$/1 μM (±)-propranolol at room temperature. When [^{3}H]DHA was used, 150–200 μg of membrane protein were used and the wash buffer was ice-cold without propanolol. For all assays, nonspecific binding was determined in the presence of 2 μM (±)-propranolol and routinely represented less than 10% of the total bound radioligand.

Assay of adenylate cyclase. Reaction mixtures contained membranes or lysates (15–30 μg of protein), 0.1 M Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.2 mM [α-^{32}P]ATP (1–2 μCi), 1 mM cyclic AMP, 1 mM EDTA, 20 mM phosphocreatine, 20 μg of creatine phosphokinase, and various effectors in a final volume of 0.1 ml. The reactions were incubated at 30° for 10 min, and ^{32}P-labeled cyclic AMP was isolated by the method of Salomon *et al.* (22). Basal activity was determined in the presence of 10 nM GTP and 2 μM (±)-propranolol (the latter was added in case any ISO was still present in preparations from ISO-treated cells). ISO-stimulated activity was measured with 10 nM GTP and 1 μM ISO. NaF was used at a concentration of 10 mM.

Other methods. Intracellular cyclic AMP accumulation was determined by incubating intact cells in multicluster trays in Dulbecco's modified Eagle's medium buffered with 25 mM Hepes and containing 1 mM IBMX at 37° for 10 or 20 min in the presence and absence of 10 μM ISO (6, 21). Cyclic AMP was extracted from the cells with 0.1 M HCl and determined by radioimmunoassay using antibodies from Collaborative Research (Waltham, Mass.) and [^{125}I]-2′-*O*-succinyl(iodotyrosine methyl ester)-cyclic AMP from Meloy Laboratories (Springfield, Va.). Protein was determined as described previously (6).

Presentation of data. Each experiment was carried out at least three times unless otherwise stated, and representative experiments are presented in the tables and figures. All values represent the mean of triplicate determinations, and standard deviations were routinely less than 5% of the mean.

RESULTS

Desensitization and down-regulation as measured on intact cells. When rat glioma C6 cells were exposed to ISO, there was a time- and dose-dependent loss of agonist-stimulated cyclic AMP production and [^{125}I]IPIN binding sites (Fig. 1). The former appeared to occur more rapidly and extensively and at lower ISO concentrations than did the latter. As the cells quickly became desensitized, we rechallenged them with ISO for only 10 min, but similar results were obtained using a 20-min incubation (data not shown). In order to prevent any further changes during the long binding assay, we measured binding to intact cells at 4° instead of 22° (20). Whereas cells exposed to 1 μM ISO for 2 hr at 37° exhibited a loss of both responsiveness and receptors (Fig. 1), no loss was observed at 4° (Table 1). The time course of [^{125}I]IPIN binding at 4° to control and ISO-treated (1 μM for 1 hr) cells was identical over a 6-hr period, and binding appeared to reach equilibrium by then; at each of the times tested (0.5, 1, 2, 3, 4, and 6 hr), the ratio of antagonist bound to ISO-treated and control cells was the same, 0.755 ± 0.014. Based on Scatchard analysis, there appeared to be only a single class of receptors on control and desensitized intact cells, with K_d values of 133 and 129 pM, respectively. Thus, differences in the number of binding sites did not appear to reflect differences in rate of binding or in affinity of the antagonist. Therefore, we routinely measured binding at 4° for 2 hr with 200 pM [^{125}I]IPIN. Other agents that elevate cyclic AMP levels also caused a loss *beta*-receptors as measured on intact cells (Table 2). Whereas an overnight exposure to ISO eliminated most of the receptors, the other agents were not as effective.

When cells treated with 0.1 μM ISO for 2 hr were washed and challenged with increasing concentrations of agonist, the K_{act} was 3-fold higher as compared with that for control cells (Fig. 2A). In contrast, cells treated with 1 mM IBMX for 20 hr had a K_{act}, similar to that of control cells even though their maximal responsiveness and *beta*-receptor number had decreased to levels found in the ISO-treated cells (Fig. 2B). The K_{act} values for ISO in three separate experiments were as follows: control, 5.5 ± 0.9 nM; ISO-treated, 16.2 ± 0.3 nM; and IBMX-treated, 6.7 ± 3.3 nM.

Desensitization and down-regulation as measured in lysates and membranes. When cells exposed to various effectors were washed, lysed, and assayed for *beta*-receptors, results similar to those obtained with intact cells

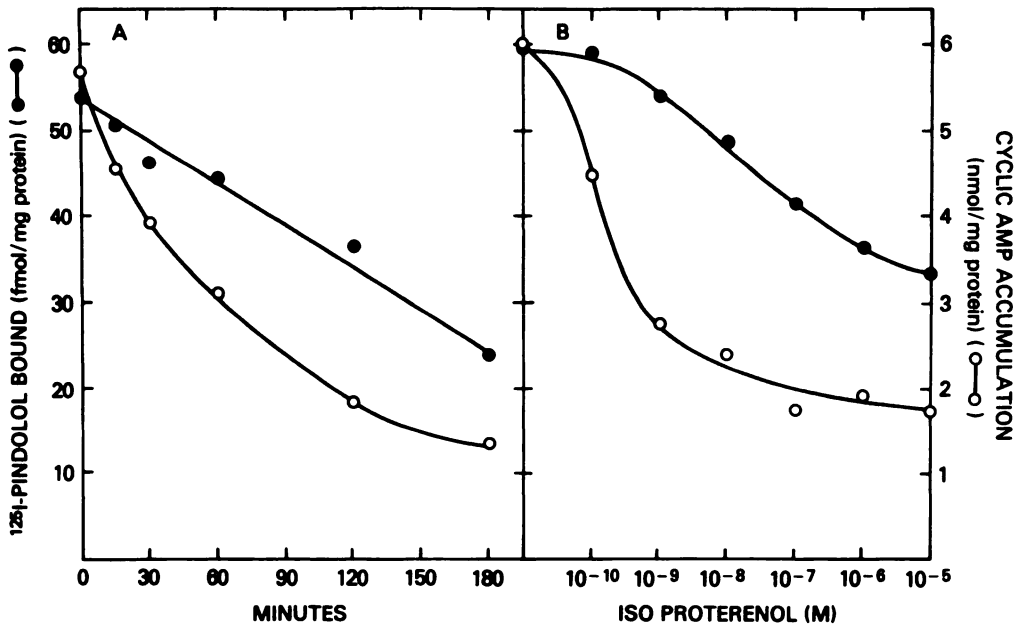

FIG. 1. *Effect of ISO on loss of beta-receptors and agonist-stimulated cyclic AMP production in intact rat glioma C6 cells*

A. Cells were incubated with 0.1 μM ISO for the times indicated, washed, and assayed for specific [^{125}I]IPIN binding at 4° for 2 hr (●) and cyclic AMP accumulation following a rechallenge for 10 min with 10 μM ISO (○) as described under Experimental Procedures. B. Same as in A except that the cells were exposed to the indicated concentrations of ISO for 2 hr.

TABLE 1

Effect of low temperature on ISO-induced loss of responsiveness and beta-receptors in intact rat glioma C6 cells

Cells were incubated for 2 hr at 4° in the presence and absence of 1 μM ISO. The cells then were washed and assayed for ISO-stimulated cyclic AMP production at 37° (20 min with 10 μM ISO) and [^{125}I]IPIN binding at 4° for 2 hr as described under Experimental Procedures. Cells incubated at 37° for 2 hr with 1 μM ISO lost 70% of their responsiveness and 48% of their receptors.

Treatment	Cyclic AMP accumulation	*Beta*-receptors
	nmol/mg protein	*fmol/mg protein*
Control	10.9	46.9
ISO	10.6	46.6

TABLE 2

Effect of various agents on beta-receptors of intact rat glioma C6 cells

Cells were incubated with various agents indicated below, washed, and assayed for specific [^{125}I]IPIN binding at 4° for 2 hr as described under Experimental Procedures. Values are the means ± standard deviation for triplicate determinations from a representative experiment. Similar results were obtained in two additional experiments.

Treatment	[^{125}I]IPIN specifically bound
	fmol/mg protein
Control	44 ± 4
ISO, 1 μM for 2 hr	28 ± 1
ISO, 1 μM for 20 hr	3 ± 1
Choleragen, 10 nM for 20 hr	21 ± 3
Dibutyryl cyclic AMP, 1 mM for 20 hr	24 ± 2
IBMX, 1 mM for 20 hr	18 ± 2
Dibutyryl cyclic AMP + IBMX, 1 mM each for 20 hr	12 ± 2

were observed (Table 3). We also observed a 45–55% loss of receptors in lysates from cells exposed to 1 mM 8-bromo-cyclic AMP for 20 hr. Thus, the change in receptor number was not due to release of butyrate from dibutyryl cyclic AMP (23). Crude plasma membranes prepared from the cells also gave results comparable to those obtained with intact cells and lysates (Table 3). Figure 3 shows a time course for down-regulation as measured in cell lysates. Again, agonist-mediated loss was more rapid and extensive than that caused by a combination of dibutyryl cyclic AMP and IBMX. In some experiments, there was a lag of several hours before any significant loss of receptors was observed in cells exposed to the cyclic AMP derivative.

The affinity of *beta*-receptors for ISO was determined by its ability to compete for the binding of labeled antagonists to the membranes. Consistent with other studies (3, 4, 6, 8), receptor affinity for agonist was reduced 10-fold by GTP (Fig. 4A). Receptor affinity for agonist was 4.2-fold lower for receptors from ISO-treated cells (Fig. 4B) but was actually 3-fold higher for receptors from cells treated with dibutyryl cyclic AMP and IBMX (Fig. 4C). Similar results were obtained in a second experiment using [^{3}H]DHA; shifts of 3.4-fold lower and 2.4-fold higher were observed, respectively. Dibutyryl cyclic AMP and IBMX by themselves were slightly less effective than together; the shifts were 1.8- and 1.7-fold higher, respectively. Membranes from cells treated overnight with choleragen also exhibited a higher affinity for ISO, i.e., 2-fold in two experiments. In a number of experiments, especially those using (±)-iodocyanopindolol and (±)-iodohydroxybenzylpindolol, the shift in receptor affinity for agonist in preparations from ISO-treated cells was smaller, 1.52 ± 0.125-fold (n = 7), but still significant. In other experiments with (−)-iodocya-

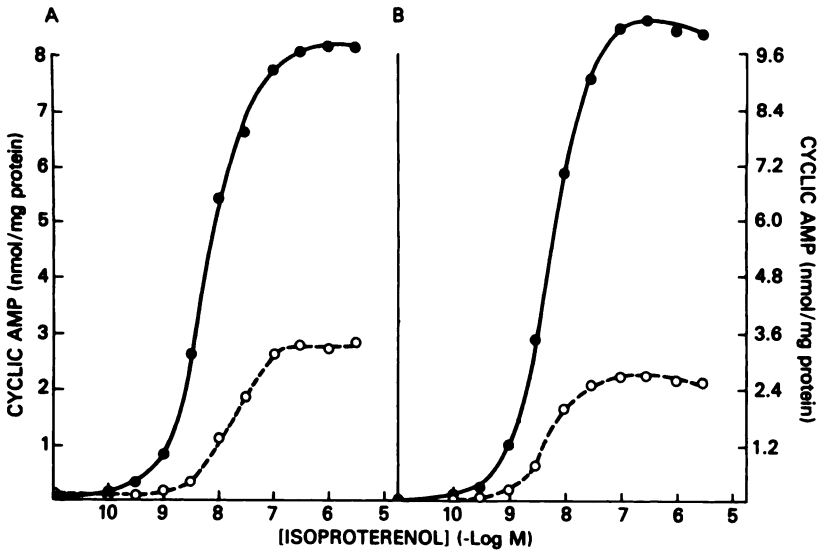

FIG. 2. *Effect of prior treatment of rat glioma C6 cells with ISO or IBMX on subsequent dose response to ISO*

A. Cells were incubated with (O) and without (●) 0.1 μM ISO for 2 hr, washed, rechallenged for 20 min with the indicated concentrations of ISO, and assayed for intracellular cyclic AMP as described under Experimental Procedures. B. Same as in A except that cells were incubated with (O) and without (●) 1 mM IBMX for 24 hr.

TABLE 3

Beta-receptors in lysates and membranes of rat glioma C6 cells: effect of prior treatment with ISO, dibutyryl cyclic AMP or IBMX

Cells were incubated with the various agents as indicated below and washed. Cell lysates and membranes were prepared and assayed for specific (−)-iodocyanopindolol binding (150 pM for 40 min at 30°) as described under Experimental Procedures.

Treatment	*Beta*-receptors	
	Lysates	Membranes
	fmol/mg protein	
None	77	423
ISO, 1 μM for 1 hr	45	224
ISO, 1 μM for 2 hr	31	204
Dibutyryl cyclic AMP, 1 mM for 20 hr	36	237
+ 1 mM IBMX	29	216

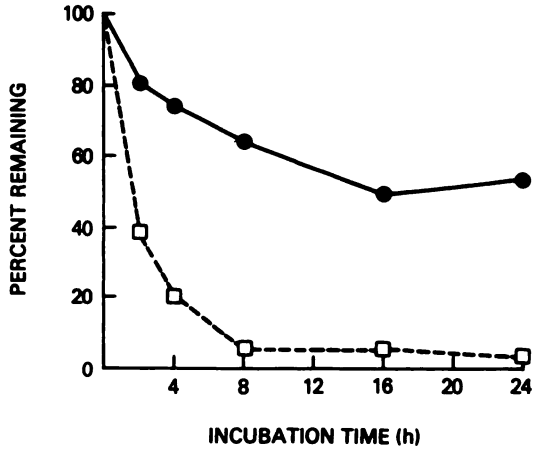

FIG. 3. *Time course for loss of beta-receptors in rat glioma C6 cells exposed to ISO or dibutyryl cyclic AMP*

Cells were incubated with either 1 μM ISO (□) or 1 mM each of dibutyryl cyclic AMP and IBMX (●) for the indicated times. The lysates then were prepared and assayed for *beta*-receptors as described under Experimental Procedures.

nopindolol, shifts of between 3- and 10-fold were consistently observed. We directly compared findings from C6 cells with those from S49 lymphoma cells using (±)-iodohydroxybenzylpindolol as the radiolabeled antagonist. When the C6 cells were treated with 1 μM ISO for 30 min, the IC_{50} for ISO was shifted 1.4-fold to the right. In the same experiment with S49 cells treated with 1 μM ISO for 15 min, the IC_{50} exhibited a 4-fold shift to the right, which is in good agreement with the 3.8-fold shift reported by others using the same cells, radioligand, and incubation conditions (8). Most likely the variation in shifts that we observed is due to the variable presence of endogenous guanine nucleotides bound to the membranes prepared from C6 cells.

Effects on adenylate cyclase activity. Previously, it had been shown that ISO-treated C6 cells exhibited a reduction in ISO-stimulated adenylate cyclase activity (6). In these studies, desensitization appeared to be homologous, since the enzyme remained completely responsive to other effectors such as NaF, guanine nucleotides, and cholera toxin (6). Cells exposed overnight to dibutyryl cyclic AMP and IBMX also exhibited a reduction in ISO- but not NaF-stimulated activity (Table 4). The loss of agonist-stimulated activity was less than the loss in *beta*-receptors, whereas for membranes from ISO-treated cells the loss of activity was always greater than the loss in receptors (Table 4). As shown in Table 5, cells exposed to either ISO or dibutyryl cyclic AMP accumulated large amounts of cyclic AMP; only the former agent, however, caused a loss of *beta*-receptors and agonist-stimulated adenylate cyclase activity within this time. As was noted with intact cells, the K_{act} for ISO-stimulated adenylate cyclase activity was increased 3- to 4-fold in membranes from cells treated with ISO but remained unchanged in membranes from cells exposed to dibutyryl cyclic AMP and IBMX.

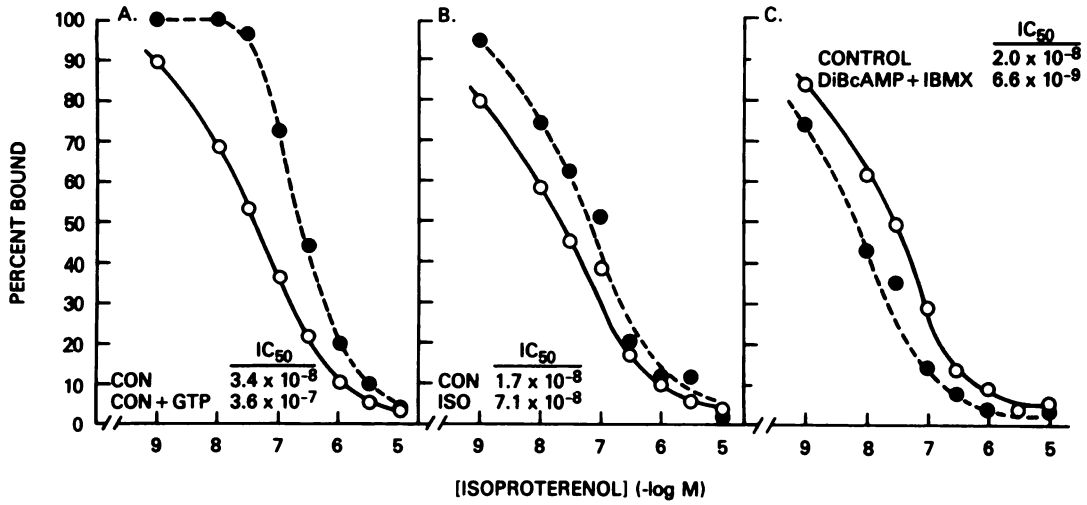

FIG. 4. *Affinity of beta-receptors for ISO in membranes from rat glioma C6 cells*

Membranes were prepared from cells treated with no addition, 0.1 μM ISO for 2 hr, or 1 mM each of dibutyryl cyclic AMP and IBMX for 18 hr and assayed for [^{3}H]DHA binding (0.8–1.0 nM for 1 hr) in the presence of increasing concentrations of ISO. A. Competition binding to membranes from untreated cells in the absence (○) and presence (●) of 0.1 mM GTP. B. Competition binding to membranes from untreated (○) and ISO-treated (●) cells. C. Competition binding to membranes from untreated cells (○) and cells treated with dibutyryl cyclic AMP and IBMX (●).

TABLE 4

Effect of prior treatment of rat glioma C6 cells with ISO or dibutyryl cyclic AMP on adenylate cyclase activity and beta-receptors

Cells were incubated as indicated below and washed. Membranes were prepared and assayed for adenylate cyclase activities and specific (−)-iodocyanopindolol binding as described under Experimental Procedures.

Treatment	Adenylate cyclase activity			*Beta*-receptors
	Basal	ISO	NaF	
	(pmol/10 min) mg^{-1} protein			*fmol/mg protein*
None	167	1150	733	386
ISO, 1 μM for 1 hr	133	592	701	205
ISO, 1 μM for 2 hr	134	460	685	161
Dibutyryl cyclic AMP, 1 mM for 20 hr + 1 mM IBMX	130	711	702	207
	129	585	787	155

Effects of cycloheximide on desensitization and down-regulation. When C6 cells were exposed to cycloheximide (10 μg/ml), protein synthesis was effectively blocked. Incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble material during a 1-hr pulse was 4.9% of control cells after a 24-hr exposure to the drug. Even after 1 and 4 hr of treatment, incorporation was 6.9% and 6.3% of control cells, respectively. Prior treatment of the cells with cycloheximide, however, did not prevent ISO-mediated desensitization as measured in intact cells (Fig. 5). Similar results were obtained when ISO-stimulated adenylate cyclase activity was assayed in membranes prepared from the cells (Fig. 6A). Cycloheximide was very effective, however, in reducing the loss of *beta*-receptors induced by the agonist (Fig. 6B). Cells treated with cycloheximide for 4 hr and then for 20 hr with 1 μM ISO in the presence of the drug lost only 58% of their receptors as compared with 98% for cells not treated with cycloheximide.

Cycloheximide also prevented down-regulation induced by dibutyryl cyclic AMP and IBMX (Table 6). In the presence of the drug, the cells retained 80–90% of both their *beta*-receptors and their ISO-stimulated adenylate cyclase activity. Thus, the loss in activity observed in C6 cells incubated with dibutyryl cyclic AMP may be due entirely to the loss of *beta*-receptors. Cycloheximide did not prevent the decrease or increase in agonist affinity for *beta*-receptors from cells treated with ISO or with

TABLE 5

Effect of ISO and dibutyryl cyclic AMP on cyclic AMP accumulation, desensitization, and down-regulation in rat glioma C6 cells

Cells were incubated with no addition, 1 μM ISO, or 1 mM dibutyryl cyclic AMP for 1 hr. Cells either were assayed for cyclic AMP accumulation or membranes were prepared and assayed for adenylate cyclase activity and specific (−)-iodocyanopindolol binding as described under Experimental Procedures.

Treatment	Cyclic AMP accumulation	Adenylate cyclase activity		*Beta*-receptors
		basal	ISO	
	pmol/mg protein	*(pmol/10 min) mg^{-1} protein*		*fmol/mg protein*
None	21	139	817	318
ISO	570	130	500	135
Dibutyryl cyclic AMP	2565	157	902	314

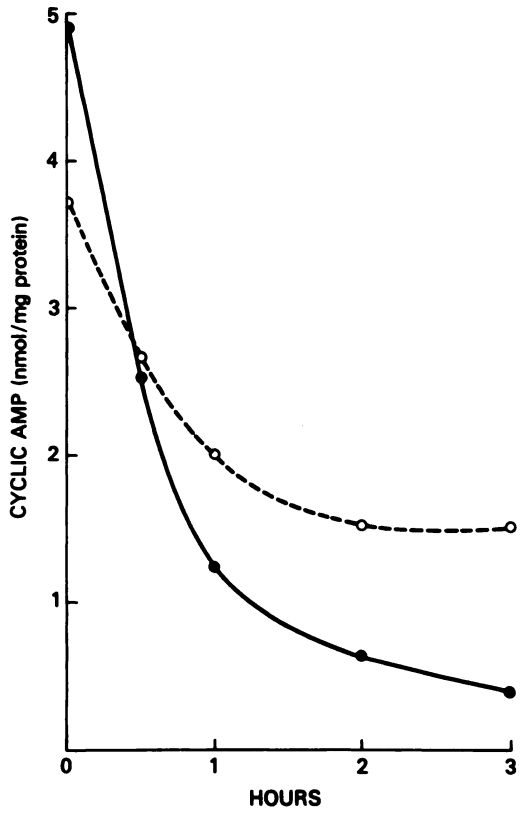

FIG. 5. *Effect of cycloheximide on ISO-mediated desensitization of rat glioma C6 cells.*

Cells were incubated with (O) and without (●) cycloheximide (10 μg/ml) for 20 hr, exposed to 0.1 μM ISO for the times indicated, washed, and assayed for cyclic AMP accumulation after a 20-min rechallenge with 10 μM ISO as described under Experimental Procedures.

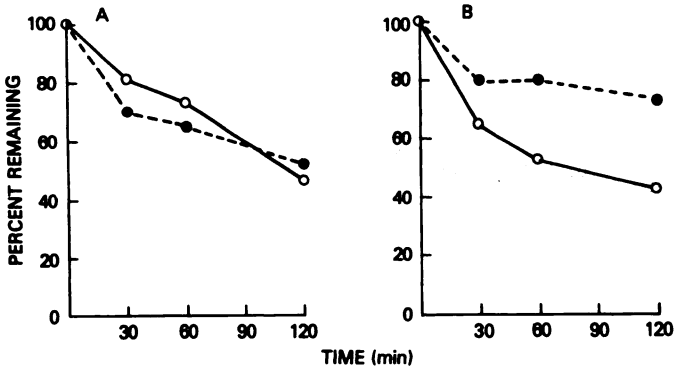

FIG. 6. *Effect of cycloheximide treatment of rat glioma C6 cells on desensitization of adenylate cyclase and loss of beta-receptors*

Cells were incubated with (●) and without (O) cycloheximide (10 μg/ml) for 16 hr and then with 1 μM ISO for the indicated times. Membranes were prepared and assayed for (A) ISO-stimulated adenylate cyclase activity and (B) specific (−)-iodocyanopindolol binding (112 pM for 40 min) as described under Experimental Procedures.

dibutyryl cyclic AMP, respectively. The affinity of the receptors for ISO was not affected significantly by prior treatment of the cells with cycloheximide, and the affinity was still modulated by GTP (data not shown).

DISCUSSION

When we exposed rat glioma C6 cells to various agents that elevated intracellular cyclic AMP levels, we observed a time-dependent loss in *beta*-adrenergic receptors. This loss was observed whether binding was measured on intact cells, cell lysates, or partially purified plasma membranes. We measured binding of [^{125}I]IPIN to intact cells at 4° in order to minimize any changes in the receptors due to further down-regulation, recycling, or biosynthesis. Recent studies (24–26) have indicated that exposure of C6 cells to ISO results in a shift of *beta*-receptors from the plasma membrane to a lighter density membrane fraction. The receptors in the latter fraction appear to be intravesicular or sequestered and are not readily accessible to hydrophilic agonists or antagonists when binding is measured at 37°. These receptors do not appear to recycle to the cell surface at 4°. In addition, binding of [^{125}I]IPIN to intact cells at 4° appears to label predominantly surface receptors, whereas binding of the various pindolol derivatives or DHA to lysates at 30° appears to label all of the receptors. Thus, our results are consistent with a loss of receptors not only from the cell surface but also from the cell. In this regard, our results agree with those of Moylan *et al.* (18), who reported that cholera toxin and dibutyryl cyclic AMP caused receptor loss in rat glioma C6-2B cells even though binding was measured at 22°, a temperature that would have permitted detection of recycling receptors had they been internalized or "sequestered." Since previous reports from that laboratory have implicated cyclic AMP as a mediator of refractoriness in C6-2B cells (14–16), we compared the effects of the agonist ISO with those of other agents on both desensitization of adenylate cyclase and down-regulation of *beta*-receptors.

Although C6 cells exposed to dibutyryl cyclic AMP and/or IBMX for 16–24 hr exhibited a reduction in ISO-stimulated cyclic AMP accumulation and adenylate cyclase activity, these treatments did not appear to cause a desensitization analogous to that caused by the agonist. Whereas ISO-treated cells exhibited a 3-fold increase in the K_{act} for ISO stimulation of cyclic AMP production or adenylate cyclase activity, no shift was observed in cells treated with IBMX or dibutyryl cyclic AMP. Similar increases in K_{act} had been observed in C6 cells (5) and S49 cells (9) following agonist-mediated desensitization. Consistent with previous studies (3, 4, 6, 8), the affinity of the *beta*-receptor for the agonist was reduced following treatment of the cells with ISO. Although the extent of the affinity shift varied among experiments, it was always significant and in the same direction as that caused by guanine nucleotides. In contrast, *beta*-receptors from cells treated with dibutyryl cyclic AMP, IBMX, or cholera toxin actually exhibited a higher affinity for ISO as determined by competition binding assays. Although we have no explanation for the apparent increase in affinity, it is inconsistent with current mechanisms of desensitization. It has been proposed that the reduction in agonist affinity as measured by changes in the IC_{50} or the K_{act} represents a functional uncoupling of the desensitized receptor from the regulatory component of adenylate cyclase (3–6, 8, 9, 25).

The time course for ISO-mediated down-regulation was much more rapid than that caused by dibutyryl cyclic AMP. In this regard, cells treated with either agent for

TABLE 6

Effect of cycloheximide on dibutyryl cyclic AMP-mediated loss of beta-receptors in rat glioma C6 cells

Cells were incubated with and without cycloheximide (10 μg/ml) for 4 hr and then for an additional 16 hr with and without 1 mM each of dibutyryl cyclic AMP and IBMX. Membranes were prepared and assayed for adenylate cyclase activity and specific (−)-iodocyanopindolol binding as described under Experimental Procedures. Values are the mean ± standard deviation of triplicate determinations from a representative experiment.

Cycloheximide	Dibutyryl cyclic AMP + IBMX	Adenylate cyclase activity		*Beta*-receptors[a]
		basal	ISO	
		(pmol/10 min) mg^{-1} protein		*fmol/mg protein*
−	−	103 ± 2	415 ± 6	294 ± 15
−	+	113 ± 3	300 ± 15	186 ± 5
+	−	90 ± 1	448 ± 22	259 ± 12
+	+	82 ± 5	415 ± 22	214 ± 3

[a] Similar effects of cycloheximide on receptor loss were observed in two additional experiments. As a percentage of control, specific binding values were 44 and 52% without the drug and 82 and 92% with the drug.

1 hr exhibited a large increase in cyclic AMP levels, yet only the agonist-treated cells were down-regulated as well as desensitized. After 20 hr, cells incubated with ISO had lost most of their *beta*-receptors and agonist-stimulated adenylate cyclase activity. Cells exposed to dibutyryl cyclic AMP for 20 hr had lost approximately 50% of their receptors and even less of their cyclase activity. When conditions were chosen so that the loss of receptors was similar, ISO induced a larger reduction in agonist-stimulated activity than did dibutyryl cyclic AMP. This is consistent with the latter agent's causing a reduction in activity only through loss of receptors.

The results that we obtained with rat glioma C6 cells are very different from those found with avian erythrocytes (11–13). In the latter cells, both ISO and cyclic AMP caused desensitization of adenylate cyclase but without any loss of *beta*-receptors. The type of desensitization apeared to be heterologous, as NaF- and guanine nucleotide-stimulated activities also were reduced. Finally, agonist affinity for *beta*-receptors was reduced after desensitization by ISO or by cyclic AMP derivatives (13).

We also were able to separate the effects of ISO and dibutyryl cyclic AMP by inhibiting protein synthesis with cycloheximide. In agreement with Homburger *et al.* (5), we observed that the drug did not prevent ISO-mediated desensitization but did inhibit *beta*-receptor loss.[3] Cycloheximide was even more effective in blocking down-regulation by dibutyryl cyclic AMP and did inhibit the reduction in agonist-stimulated adenylate cyclase activity. We believe that these data support our contention that the latter reduction is due to loss of receptors in cells exposed to dibutyryl cyclic AMP.

[3] After we had submitted our manuscript, a paper appeared by Federich *et al.* (25), which described ISO-mediated desensitization in rat glioma C62B cells. They also found a more rapid loss of ISO-stimulated adenylate cylase activity than of *beta*-adrenergic receptors in cell lysates. Prior treatment of the cells with cycloheximide inhibited receptor loss but not desensitization of the agonist-stimulated cyclase. As we had found, the drug appeared to reduce the loss of responsiveness when cyclic AMP accumulation in whole cells was measured. They did not address the role of cyclic AMP in desensitization and down-regulation.

We conclude that *beta*-adrenergic agonists cause both desensitization of the agonist-stimulated adenylate cyclase and down-regulation of *beta*-adrenergic receptors in rat glioma C6 cells. Desensitization appears to involve a functional uncoupling of the receptor from the regulatory component of the cyclase, does not require protein synthesis, and is not mimicked by cyclic AMP. Down-regulation depends on protein synthesis and can be caused by other agents that elevate cyclic AMP levels in these cells. Finally, desensitization and down-regulation can occur independently of each other.

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