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Catecholamine-Induced Desensitization of Adenylate Cyclase in Rat Glioma C6 Cells

Evidence for a Specific Uncoupling of *Beta*-Adrenergic Receptors from a Functional Regulatory Component of Adenylate Cyclase

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SUMMARY

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Catecholamine-induced desensitization was compared in two strains of rat glioma C6 cells [low passage (C6LP) and high passage (C6HP)]. When exposed to isoproterenol, C6LP cells accumulated high levels of cyclic AMP (3–4 nmoles/mg of protein), whereas C6HP cells did not. After prolonged exposure to isoproterenol, both C6LP and C6HP cells exhibited a diminished response when rechallenged with the agonist. The desensitization process was both time- and dose-dependent and similar parameters were observed for C6LP and C6HP cells. C6HP cells exposed to isoproterenol in the presence of a phosphodiesterase inhibitor initially accumulated large amounts of cyclic AMP, but the inhibitor did not alter the time course of agonist-induced desensitization. In addition, the inhibitor, which by itself elevated cyclic AMP levels more than did isoproterenol by itself in C6HP cells, did not induce a refractory state. Agonist-induced loss in *beta*-adrenergic receptors was much slower than the rate of desensitization. Membranes from desensitized cells, however, exhibited an apparent lower affinity for agonist as measured by agonist displacement of labeled antagonist binding. A substantial loss of isoproterenol-stimulated adenylate cyclase activity in membranes prepared from agonist-treated C6LP and C6HP cells was observed without a significant loss in NaF-stimulated activity. Isoproterenol-treated C6HP cells remained completely responsive to cholera atoxin. Desensitized C6LP cells exhibited a reduced response to the toxin, which was partially overcome by the phosphodiesterase inhibitor. Activation of cyclase, however, was not reduced in either C6LP or HP cells treated with agonist and then toxin. Furthermore, cyclase was activated to the same extent when membranes from control or desensitized cells were incubated with the A_1 subunit of cholera toxin and NAD. We conclude that the large accumulation of cyclic AMP in C6LP cells exposed to *beta*-agonists does not mediate the subsequent desensitization process. Instead, the initial stages of catecholamine-induced desensitization in both C6LP and C6HP cells appear to represent a specific uncoupling of the *beta*-adrenergic receptor from a functional regulatory component of adenylate cyclase.

INTRODUCTION

The responsiveness of cells to *beta*-adrenergic agonists diminishes with prolonged exposure to these agents. This phenomenon, referred to here as desensitization, has been examined in a number of cell lines, and loss of catecholamine-stimulated adenylate cyclase activity has been observed with and without a corresponding loss of *beta*-adrenergic receptors (1–5). Rat glioma C6 cells represent those cells that become desensitized without any substantial decrease in *beta*-receptor number (5, 6). Some investigators, using different cell lines, have proposed a model of catecholamine-induced desensitization that is specific and involves "uncoupling" of *beta*-receptors from adenylate cyclase (7–9). Terasaki *et al.* (5), de Vellis and

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Brooker (10), and Nickols and Brooker (11, 12) have proposed that a component mediating catecholamine refractoriness in rat glioma C6-2B cells is controlled by the agonist-stimulated increase in intracellular cyclic AMP. In support of this hypothesis, they reported that prior treatment of C6-2B cells with CT,[2] which also elevates cyclic AMP levels, led to a loss of ISO responsiveness and that ISO-desensitized cells were refractory to CT (11).

A variant of rat glioma C6 cells (C6HP) which does not accumulate cyclic AMP when exposed to ISO or CT unless phosphodiesterase inhibitors are present has recently been described (13).[3] The availability of C6HP as well as C6LP cells, which appear to be similar to C6-2B cells in their ability to accumulate cyclic AMP when exposed to ISO or CT, provided an opportunity to reexamine the interrelationship between CT and ISO action on these cells and the role of cyclic AMP in the desensitization process.

MATERIALS AND METHODS

Materials. (−)-[Propyl-1,2,3-^{3}H]dihydroalprenolol (37–43 Ci/mmole) and [2,8-^{3}H]adenosine 3′,5′-cyclic phosphate, ammonium salt (32 Ci/mmole), were purchased from New England Nuclear Corporation (Boston, Mass.). ISO was purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisc.) and CT was obtained from Schwarz/Mann (Orangeburg, N. Y.). ATP, GTP, Gpp(NH)p, MIX, and mepacrine (quinacrine) were obtained from Sigma Chemical Company (St. Louis, MO.). The ganglioside G_{M1} was purified from bovine brain (14). All other chemicals were of analytical grade. Culture media were obtained from Microbiological Associates (Bethesda, Md.).

Cells and cell culture. Rat glioma C6 cells were obtained at Passage 39 from the American Type Culture Collection (Rockville, Md.). The cells were cultured in Dulbecco's modified Eagle's medium containing 0.45% glucose and supplemented with 5% fetal calf serum, 4 mM L-glutamine, and 0.05% gentamicin in a humidified atmosphere of 5% CO_2 in air at 37°. The cells were subcultured every week and the medium was changed twice per week as described previously (6). Cultures from Passages 39–80 are referred to as C6LP cells and from 115–175 as C6HP cells. Cells used for the preparation of membranes were cultured in 75 cm^2 flasks (Falcon No. 3023) and cells used for cyclic AMP accumulation experiments were cultured in multicluster trays (6 × 35-mm diameter; Linbro No. 76-047-05).

Catecholamine-induced desensitization. ISO-induced desensitization was determined as follows. Cells were incubated in Medium 199 buffered with 25 mM Hepes with and without ISO at 37°. After the appropriate time, the medium was removed and the cells were washed three times with warm phosphate-buffered saline (pH 7.4). Fresh warm Medium 199 was added and the cells were allowed to equilibrate for 10 min at 37°. The cells were then rechallenged by incubating them at 37° with and without 10 μM ISO for 10 min, unless otherwise indicated. In some experiments, the cells were allowed to equilibrate in fresh medium for 1 hr before adding the agonist (6). Because the resensitization process is very slow (see Results), we observed no differences between a 10-min and a 1-hr equilibration period. After the rechallenge period, the medium was removed and the cells were assayed for cyclic AMP and protein content. In some experiments, MIX was included during the challenge and/or rechallenge incubation. In other experiments, the cells were treated with mepacrine (6), with G_{M1} (14, 15), or with CT (14, 15). In experiments in which cells were treated for 24 hr, serum-free growth medium was used instead of Medium 199. In addition, Dulbecco's medium buffered with 25 mM Hepes was substituted for Medium 199 in some experiments; similar results were obtained with the various media.

Assay of adenylate cyclase. After treating the cells with ISO and/or CT as described above, the cells were washed twice with ice-cold saline and once with 2 mM Tris-Cl (pH 7.4)-1 mM EDTA-0.2 mM dithiothreitol. The cells were detached by allowing them to remain in the latter buffer for 10 min at 4°. The cell suspension was agitated by using a Vortex mixer and allowed to stand for 15 min at 4° in order to lyse the cells. The cell lysate was centrifuged at 40,000 × *g* for 30 min and the pellet was suspended in lysing buffer. In later experiments, the gelated pellet of nucleoprotein was discarded at this point (the specific activity of adenylate cyclase was enriched up to 2-fold by this latter technique). The membranes were assayed for adenylate cyclase activity as described previously (14, 15) or further treated with activated CT.

CT was activated by incubating a solution containing 10 μM CT, ovalbumin (0.4 mg/ml) 10 mM dithiothreitol, 40 mM Hepes (pH 7.5), and 135 mM NaCl for 10 min at 33°. Membranes were activated with CT by incubating 50–100 μg of membrane protein with 1 μM activated CT, 2 mM NAD, and 0.1 mM GTP in a final volume of 50 μl for 10 min at 33°. The activated membranes then were directly assayed for adenylate cyclase activity (14, 15). Adenylate cyclase activity in membranes incubated with all of the above additions except CT was not increased above basal activity. Additions to the cyclase assays were as follows: 50 μM GTP (basal); 50 μM GTP and 10 μM ISO; 10 mM NaF; or 50 μM Gpp(NH)p. Values for adenylate cyclase activity are the means of triplicate determinations; standard deviations were less than 10% of the mean unless otherwise indicated. Our standard assay (14, 15) and that of Salomon *et al.* (16) gave similar results.

Binding of [^{3}H]DHA. Binding of [^{3}H]DHA was determined as described previously (6) on the same membrane fractions used for the assays of adenylate cyclase. Nonspecific binding is defined as binding in the presence of 10 μM *dl*-propranolol or 100 μM ISO. In some experiments, displacement of [^{3}H]DHA binding by increasing concentrations of ISO was measured in the presence and absence

[2] The abbreviations used are: CT, cholera toxin; ISO, (−)-isoproterenol; C6HP and C6LP cells, C6 high passage and low passage cells, respectively; Gpp(NH)p, 5′-guanylylimidodiphosphate; MIX, 3-isobutyl-1-methylxanthine; G_{M1}, galactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]galactosylglucosylceramide; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid; DHA, dihydroalprenolol.

[3] The properties of rat glioma C6LP and C6HP cells will be described in detail elsewhere. The inability of C6HP cells to accumulate cyclic AMP in response to ISO or CT is not due to a rapid excretion of cyclic AMP into the medium.

TABLE 1

Stimulation of cyclic AMP accumulation in rat glioma C6 cells by ISO and CT

Cells cultured in 35-mm wells of multicluster trays were washed with phosphate-buffered saline (pH 7.4) and then exposed to 10 μM ISO for 10 min or to 10 nM CT plus 0.01% bovine serum albumin for 90 min at 37° in 2 ml of Medium 199 buffered with 25 mM Hepes. The medium was then removed and the cells were assayed for cyclic AMP and protein content as described under Materials and Methods. Values represent the mean ± standard deviation for three separate cultures; for each culture, the cyclic AMP content was determined in duplicate. Comparable results were obtained in many additional experiments.

Cells	Intracellular cyclic AMP accumulation		
	Basal	ISO	CT
	pmoles/mg protein		
C6LP	10.9 ± 1.1	3390 ± 220	350 ± 24
C6HP	13.9 ± 1.8	15.0 ± 2.2	13.7 ± 1.7

of 100 μM GTP. Samples were assayed in triplicate and the triplicate values varied less than 10%.

Other methods. Cyclic AMP was determined as described previously (6, 14). Unless otherwise indicated, each value is the mean of triplicate determinations and the standard deviation was less than 10% of the mean. Protein was determined by the method of Lowry *et al.* (17). Binding of [^{125}I]hydroxybenzylpindolol (obtained from New England Nuclear Corporation) was assayed as described previously (18).

RESULTS

Properties of C6LP and C6HP cells. Desensitization to catecholamines was studied in two different variants of rat glioma C6 cells.[3] The C6 cells of low passage (C6LP) possessed a high capacity to accumulate cyclic AMP in response to ISO and CT (Table 1). In contrast, C6HP cells were unresponsive to these stimulators except in the presence of phosphodiesterase inhibitors (compare Table 1 with Table 2). Both variants possessed *beta*-adrenergic receptors with similar affinities (2 nM) for [^{3}H]DHA; C6LP cells, however, had several-fold more receptors than did C6HP cells (see Table 3). Both variants contained a membrane adenylate cyclase responsive to ISO, guanine nucleotides, and NaF (see Table 4) and to CT (see Table 5). Cyclic AMP phosphodiesterase activity was 2–3 times higher in homogenates of C6HP than in C6LP cells; in intact cells, the rate of cyclic AMP breakdown was 5 times faster in C6HP than in C6LP cells.[3] Thus, differences in *beta*-adrenergic receptor number and phosphodiesterase activity may explain in part the lack of responsiveness of intact C6HP cells to ISO.

Desensitization of cyclic AMP accumulation in intact cells. Both rat glioma C6LP and C6HP cells exposed to 10 μM ISO developed a time-dependent refractoriness to a rechallenge with 10 μM ISO (Fig. 1). The amount of cyclic AMP accumulated during the second incubation was substantially reduced compared with levels in control cells. When exposed to 10 μM ISO, a 50% loss of responsiveness was observed within 1 hr in both C6LP and HP cells; the rate of desensitization appeared to be slightly

TABLE 2

Effect of CT treatment on ISO-stimulated cyclic AMP accumulation in C6HP cells

C6HP cells cultured in 35-mm dishes were incubated with and without 10 nM CT for the indicated times and then incubated with and without 10 μM ISO for 10 min in the presence of 0.5 mM MIX, as described under Materials and Methods. Data are from three separate experiments.

CT treatment time	Cyclic AMP accumulation			
	Control cells		CT-treated cells	
	−ISO	+ISO	−ISO	+ISO
hr	*pmoles/mg protein*			
2.0	18.1	388	283	750
2.5	24.1	561	450	922
4.0	28.1	483	263	871

TABLE 3

Binding of [^{3}H]DHA to membranes prepared from control and ISO-treated rat glioma C6LP and C6HP cells

Rat glioma C6LP and C6HP cells were incubated at 37°C in medium containing 10 μM ISO for the indicated times. After the cells were washed extensively and lysed, membranes[a] were prepared and assayed for specific [^{3}H]DHA binding as described under Materials and Methods. The data are representative of three similar experiments.

ISO treatment time	Specific [^{3}H]DHA binding	
	C6LP cells	C6HP cells
hr	*fmoles/mg protein*	
0	417	171
2	330	130
24	218	82

[a] Homogenates of C6LP cells also exhibited increased binding of [^{3}H]DHA compared with homogenates of C6HP cells.

TABLE 4

Effect of ISO treatment on adenylate cyclase activities of rat glioma C6 cells

Rat glioma C6 cells cultured in 75 cm^2 flasks were incubated with and without 10 μM isoproterenol for 2 hr, washed extensively, and lysed. The lysates were centrifuged at 40,000 *g* for 30 min and the membranes obtained were assayed for adenylate cyclase activities as described under Materials and Methods. Values for each preparation are the mean of triplicate assays and have been corrected for zero time controls; standard deviations were less than 10% of the mean.

Cells	Treatment	Adenylate cyclase activity			
		Basal	ISO	Gpp(NH)p	NaF
		pmoles/min/mg protein			
Experiment 1[a]					
C6LP	None	13.9	48.1	—[b]	78.1
	ISO	4.2	12.4	—	71.7
C6HP	None	3.1	36.5	—	89.0
	ISO	0.7	13.0	—	112
Experiment 2					
C6LP	None	33.8	75.2	63.3	91.7
	ISO	30.0	46.2	54.6	101
C6HP	None	4.6	48.6	35.8	112
	ISO	6.7	21.4	30.8	101

[a] A separate preparation was used in each experiment. Similar results were obtained in many additional experiments, including ones in which cells were treated with ISO for 3 or 24 hr (see Table 5).

[b] Gpp(NH)p-stimulated activity was not assayed in Experiment 1, but similar results were obtained in another experiment (see Table 5).

TABLE 5

Effect of ISO treatment on activation of adenylate cyclase by CT in rat glioma C6 cells

Rat glioma C6 cells cultured in 75 cm^2 flasks were incubated with 10 μM ISO for the indicated times and with 0.5 μM G_{M1} for the last 2 hr of exposure to ISO. The cells then were washed extensively, incubated for 1 hr with 20 nM CT as indicated, and assayed for adenylate cyclase activity as described under Materials and Methods. In addition, membranes were incubated with activated CT (A_1) and NAD prior to the assay for cyclase activity. Similar results were obtained in three additional experiments; activation of adenylate cyclase by CT in ISO-treated cells was never less than 86% of that observed in control cells.

Cells	ISO treatment time	Adenylate cyclase activity			
		Basal	ISO	CT	A_1 + NAD
	hr	*pmoles/min/mg protein*			
C6LP[a]	0	23.4	72.6	120	164
	3	23.9	41.0	103	162
C6HP[b]	0	14.6	108	104	98.7
	3	13.9	44.5	96.3	94.7
	24	15.2	25.7	111	94.3

[a] NaF-stimulated activities for control cells and cells treated for 3 hr with ISO were 158 and 164; Gpp(NH)p-stimulated activities were 114 and 103, respectively.

[b] NaF-stimulated activities for cells treated 0, 3, and 24 hr with ISO were 229, 212, and 223, respectively; corresponding values for Gpp(NH)p-stimulated adenylate cyclase activity were 137, 106, and 111 pmoles/mg of protein per minute, respectively.

more rapid in the latter cells. In addition, C6HP cells exhibited a similar time course of desensitization whether they were treated with ISO in the presence or absence of MIX (Fig. 1). Under the former conditions, C6HP cells initially accumulated large amounts of cyclic AMP. More extensive desensitization was observed after a 24-hr exposure to 10 μM ISO; residual sensitivity was 2.4 and 6.7% in C6LP and C6HP cells, respectively. Reversal of the desensitization process was much slower than its onset. Even after 24 hr in the absence of agonist, cells treated with ISO (10 μM for 2 hr) had regained only 60–70% of their sensitivity to ISO.

When the concentration of ISO was varied during the first incubation, the loss of catecholamine responsiveness was dose-dependent (Fig. 2). At 2 hr, a 50% loss was observed at 0.9 nM in C6LP cells and at 2 nM in C6HP cells; in separate experiments, values of 3 and 10 nM were obtained for C6LP and C6HP cells, respectively. These values are similar to the K_a values (3 nM C6LP and 4 nM for C6HP) for stimulation of cyclic AMP accumulation by the agonist in these cells.[3] After a 3-hr exposure to ISO, half-maximal desensitization occurred at 1 nM for C6LP and at 3 nM for C6HP cells. When the C6HP cells were incubated for 24 hr with varying concentrations of ISO, 50% desensitization occurred at a much lower dose of agonist, 0.2 nM compared with 4 nM at 3 hr in the same experiment. Similar results had been reported for human astrocytoma cells (7).

Since the C6HP cells were rechallenged in the presence of MIX, we examined the effects of this phosphodiesterase inhibitor on C6LP cells during the rechallenge (data not shown but see Table 6 for a comparable experiment). Although the over-all levels of cyclic AMP were higher in the presence of the inhibitor,[3] the extent of desensitization was still very high (78%) and similar to that observed in C6HP cells. Since MIX by itself elevated cyclic AMP levels in C6HP cells,[3] we compared the ability of this agent with that of ISO to induce desensitization (Table 7). Even though cyclic AMP levels in the MIX-treated cells increased over 5-fold after 10 min and were still twice basal after 3 hr, their subsequent response to ISO was not significantly different from that of naive cells. In contrast, there was no significant increase in cyclic AMP in ISO-treated cells; yet, when rechallenged with the agonist, cyclic AMP accumulation was less than 10% of that observed in naive cells.

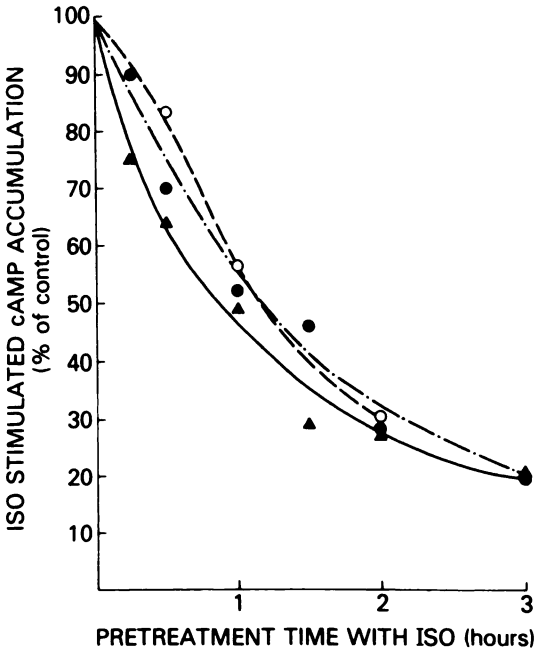

FIG. 1. *Time course of loss of ISO-stimulated cyclic AMP accumulation in rat glioma C6 cells exposed to ISO*

C6LP (○) and C6HP (●, ▲) cells were incubated with and without 10 μM ISO for the indicated times, washed extensively, incubated for 10 min with and without 10 μM ISO, and analyzed for intracellular cyclic AMP (*cAMP*) levels as described under Materials and Methods. In addition, C6HP cells (●) were incubated as above in medium containing 1 mM MIX; both sets of C6HP cultures were rechallenged in the presence of 1 mM MIX. Values are the mean of triplicate determinations and are expressed as the percentage of ISO-stimulated cyclic AMP produced in treated cultures compared with control cultures during the rechallenge incubation. Control values (with and without ISO during the rechallenge) were 2925 and 12.3 (○), 294 and 21.3 (●), and 271 and 23.3 (▲) pmoles/mg of protein.

Effect of desensitization on beta-adrenergic receptors. We next examined whether prolonged exposure of rat glioma C6 cells to *beta*-agonists affected the number of *beta*-receptors. C6 cells were incubated with and without 10 μM ISO for 2 hr and washed extensively. Binding of [^{3}H]DHA to membranes prepared from the cells was then determined (Table 3). There was only a modest reduction (20–24%) in the number of specific binding sites in the ISO-treated cells. As previously reported (6), the affinity of the labeled antagonist for *beta*-receptors of desensitized cells was not altered from that of control cells (1.5–2.0 nM). When the cells were exposed to ISO for 24 hr, they exhibited a more substantial reduction (~50%) in specific ligand binding (Table 3). Thus, the

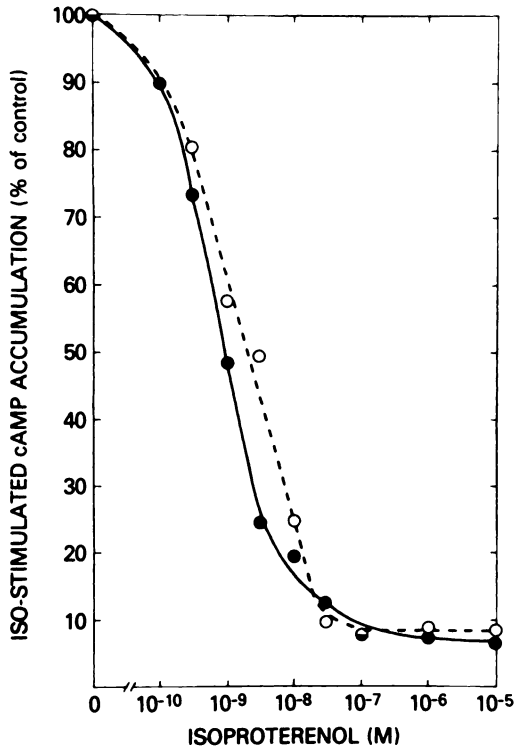

FIG. 2. *Desensitization of rat glioma C6 cells as a function of ISO concentration*

C6LP (●) and C6HP (○) cells were incubated for 2 hr with the indicated concentrations of ISO, washed extensively, rechallenged with 10 μM ISO for 10 min, and assayed for cyclic AMP (*cAMP*) content as described under Materials and Methods. C6HP cells were rechallenged in the presence of 1 mM MIX. Values are expressed as described in the legend to Fig. 1. Control values were 4675 and 10.4 pmoles/mg of protein for C6LP and 568 and 36.8 pmoles/mg of protein for C6HP cells.

TABLE 6

Responsiveness of ISO-desensitized rat glioma C6 cells to CT

C6 cells were incubated with and without 10 μM ISO for 3 or 24 hr, washed extensively, and then incubated for 90 min with no addition, with 10 nM CT,[a] or with 10 μM ISO (added for the final 10 min); the cells then were analyzed for cyclic AMP and protein content as described under Materials and Methods. Where indicated, 1 mM MIX was added to the final incubation.

Treatment time	Treatment	Cyclic AMP accumulation			
		MIX	Basal	ISO	CT
hr		*pmoles/mg protein*			
C6LP Cells					
3	None	−	24.2	2715	465
	ISO	−	28.7	197	79.6
3[a]	None	+	92.1	4461	2185
	ISO	+	117	1082	1297
24	None	+	35.9	7557[b]	824
	ISO	+	24.5	205[b]	517
C6HP Cells					
3[a]	None	+	34.5	589	974
	ISO	+	47.0	131	992
24	None	+	29.8	327	524
	ISO	+	34.7	54.6	507

[a] Cells were treated with G_{M1} during the 3-hr incubation and exposed to 20 nM CT for 1 hr.

[b] Cells were exposed to ISO for 20 min during the final incubation.

TABLE 7

Effects of prior incubation with MIX or ISO on subsequent responsiveness of rat glioma C6HP cells to ISO

C6HP cells were incubated with no addition, 1 mM MIX, or 10 μM ISO for 3 hr when some of the samples were analyzed for cyclic AMP content. Other samples were washed extensively and incubated for an additional 10 min in fresh medium containing 10 μM ISO, 1 mM MIX, or both. Values are the mean ± standard deviation of triplicate determinations. Similar results were obtained in a separate experiment; MIX elevated cyclic AMP levels 2-fold at 10 min and 3-fold at 3 hr but, the response to ISO during the rechallenge was 92% of that of naive cells.

Treatment	Cyclic AMP accumulation		
	Treatment time		Rechallenged with Iso + MIX
	10 min	3 hr	
	pmoles/mg protein		
None	—[a]	10.8 ± 2.7	587 ± 57.5
MIX	40.6 ± 8.1	22.2 ± 4.1	529 ± 69.8
ISO	7.8 ± 1.9	10.9 ± 1.8	50.6 ± 6.4

[a] Not determined.

time course of *beta*-receptor loss is much slower than the time course of desensitization. Additionally, we observed in membranes prepared from C6LP cells desensitized for 2 hr with 10 μM ISO that the apparent K_d for ISO displacement of labeled antagonist binding (either [^{3}H]DHA or [^{125}I]hydoxybenzylpindolol) was shifted 5-fold to the right (2×10^{-7} M in control versus 1×10^{-6} M in desensitized). Furthermore, the apparent K_d for ISO displacement of antagonist binding was shifted to the right (10-fold) in control but not desensitized membranes by the addition of GTP to the binding assays. Similar observations have been reported by others in studies using different cell lines (8, 9).

Adenylate cyclase activities in desensitized cells. Membranes were prepared from control and ISO-treated cells and assayed for adenylate cyclase activities. There was a marked reduction in ISO-stimulated activity when

TABLE 8

Effect of mepacrine on desensitization of rat glioma C6 cells by ISO

Rat glioma C6 cells were incubated with the indicated concentration of mepacrine for 2 hr in the presence and absence of 10 μM ISO. The cells then were washed extensively and incubated in fresh medium for 10 min with and without 10 μM ISO; for the rechallenge incubation, C6HP cultures contained 1 mM MIX. Data are expressed as indicated in the legend to Fig. 1 and are representative of many additional experiments with C6LP cells (see ref. 6) and of three similar experiments with C6HP cells.

Mepacrine	Cyclic AMP accumulation during rechallenge[a]	
	C6LP cells	C6HP cells[b]
mM	*% of control*	
0	13.2	32.1
0.1	77.5	40.6
0.3	83.5	37.3

[a] For control C6LP cells, cyclic AMP levels rose from 10.0 to 3890 pmoles/mg of protein during the rechallenge incubation with ISO; for C6HP cells, the corresponding values were 25.3 and 612.

[b] Mepacrine by itself at these concentrations had no effect on stimulation of cyclic AMP production by ISO and had a modest effect (2- to 4-fold increase) on basal cyclic AMP levels as previously noted for C6LP cells (6).

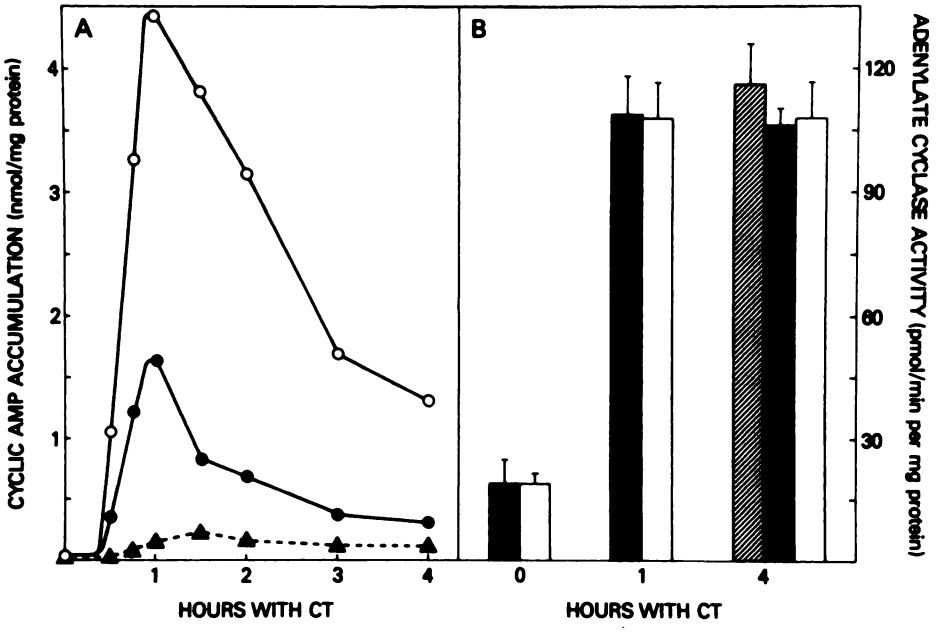

FIG. 3. *Effect of treatment of rat glioma C6LP cells with G_{M1} and MIX on time course of cyclic AMP accumulation and activation of adenylate cyclase in response to CT*

A. C6LP cells cultured in 35-mm dishes were incubated with (○, ●) and without (△) 0.5 μM G_{M1} for 1 hr at 37°, washed and incubated with 10 nM CT in the presence (○) and absence (●, △) of 0.5 mM MIX for the indicated times, and assayed for cyclic AMP content as described under Materials and Methods.

B. Cells in 75 cm^2 flasks were treated with (*solid* and *open bars*) and without (*hatched bar*) 0.5 μM G_{M1} for 1 hr at 37°. All of the cells then were washed and incubated for 4 hr in the presence (*open bar*) and absence (*solid* and *hatched bars*) of 1 mM MIX; CT (10 nM) was added at 0, 3, and 4 hr. All of the cells were then harvested and membranes were prepared and assayed for adenylate cyclase activity in the presence of 50 μM GTP as described under Materials and Methods.

agonist-treated cells were assayed (Table 4). Although there was some variation from experiment to experiment, both C6LP and C6HP cells previously exposed to ISO always exhibited a lower catecholamine-stimulated cyclase activity, whereas there was little if any loss in NaF- or Gpp(NH)p-stimulated activity. As previously noted,[3] we observed large variations in basal adenylate cyclase activity. In addition, basal activity in some experiments was substantially reduced in cells previously treated with ISO. Similar results also had been observed for rat glioma C6-2B cells (5).

Effect of mepacrine on isoproterenol-induced desensitization. Previous studies demonstrated that mepacrine, a phospholipase inhibitor, blocked catecholamine-induced desensitization in C6LP cells (6). We compared the effects of this agent on the desensitization process in C6LP and C6HP cells (Table 8). In agreement with the previous report (6), 0.1 mM mepacrine effectively blocked desensitization in C6LP cells. Mepacrine, even at 0.3 mM, had no effect on desensitization in C6HP cells. Mepacrine by itself had no effect on the ISO-stimulated levels of cyclic AMP and only a small effect on the basal levels in the latter cells (data not shown).

Effects of CT on rat glioma C6 cells. As reported elsewhere (see Table 1), C6HP cells did not accumulate cyclic AMP when exposed to CT unless MIX was present. When C6HP cells were incubated with 10 nM CT for different times and then exposed to 1 mM MIX for 10 min, cyclic AMP levels reached a maximum after 3 hr and remained unchanged for up to 8 hr. Similar results were observed when the cells were continually exposed to 1 mM MIX (data not shown). Thus, C6HP cells did not become refractory to CT irrespective of whether or not they were accumulating cyclic AMP. In addition, adenylate cyclase remained fully activated even after the cells were exposed for 2 days to CT (14). C6LP cells accumulated cyclic AMP when treated with CT; MIX increased the response.[3] Similar results had been observed with C6-2B cells (11). Prior treatment of C6LP cells with G_{M1} to increase the number of toxin receptors (14, 15) had even greater effects on cyclic AMP accumulation, and the response was further enhanced by MIX (Fig. 3A). Maximal levels were reached at 90 min in control cells and at 60 min in the G_{M1}-treated cells. Cyclic AMP levels decreased with further incubation even in the presence of MIX.[4] When adenylate cyclase activity was directly assayed, there was no evidence for any refractoriness (Fig. 3B). Thus, activities were essentially the same at 1 and 4 hr in G_{M1}-treated cells, whether MIX was present or not, and at 4 hr in cells not treated with G_{M1}.

C6HP cells were incubated with and without CT and then were incubated with and without 10 μM ISO in the presence of MIX for 10 min (Table 2). The net increase in cyclic AMP due to ISO was similar in control and CT-treated cells. When the ISO concentration was varied, the CT-treated cells were more sensitive to lower concentrations of agonist and the apparent K_a for ISO was shifted from 10 to 2–3 nM. A similar shift had been

[4] After prolonged exposure to ISO or CT, both C6LP cells and C6HP cells (in the presence of MIX) excrete cyclic AMP into the medium.

observed in C6-2B cells (11). When C6LP cells were treated first with CT and then with ISO, cyclic AMP accumulation was not additive and often lower than in cells only exposed to ISO even in the presence of MIX (data not shown). Similar results had been reported for C6-2B cells (11).

Effects of desensitization on response of rat glioma C6 cells to CT. C6HP cells were incubated with 10 μM ISO for several hours to induce desensitization and exposed to CT (Table 6). Although the ISO-treated cells were refractory to a second challenge of agonist, they remained completely responsive to CT even after a 24-hr treatment with ISO. When C6LP cells were first treated with ISO and then exposed to CT in the absence of ISO, cyclic AMP accumulation was substantially reduced in the ISO-desensitized cells (Table 6). If the cells were rechallenged in the presence of MIX, the response to CT was partially regained. Thus, cells exposed to ISO for 24 hr remained almost completely refractory to the agonist but retained 60% of their response to CT.

In order to circumvent the potential complications of induced phosphodiesterase activity as well as excretion of cyclic AMP into the medium during the long incubation periods,[4] adenylate cyclase activities were directly measured (Table 5). The results obtained with C6LP cells were comparable with those obtained with C6HP cells. ISO-stimulated activity was reduced substantially in membranes prepared from ISO-treated cells, whereas adenylate cyclase was activated to the same extent in membranes prepared from control and desensitized cells further incubated with CT. In addition, prior treatment of cells with ISO had no effect on the ability of the A_1 subunit of CT plus NAD to activate cyclase in membranes.

DISCUSSION

Rat glioma C6LP and C6HP cells exposed to the *beta*-adrenergic agonist ISO exhibited a dose- and time-dependent loss of ability to accumulate cyclic AMP upon subsequent rechallenge with this agonist. A corresponding loss of ISO-stimulated adenylate cyclase activity also was observed in the ISO-treated cells. Although extensive desensitization was obtained after a 2-hr treatment with agonist, there was only a slight decrease in the number of [^{3}H]DHA binding sites and no change in receptor affinity for this *beta*-antagonist. The number of *beta*-receptors decreased by 50% after a 24-hr exposure when desensitization was essentially total. Thus, loss of catecholamine sensitivity proceeded more rapidly than loss of *beta*-receptors in C6 glioma cells. Similar observations had been reported for human astrocytoma cells by Su *et al.* (7, 9). We also observed that the apparent affinity of the receptors for ISO was lower and was not modulated by GTP in membranes prepared from desensitized cells. Similar effects have been reported for other cell lines (8, 9).

We had demonstrated previously that C6HP cells failed to accumulate cyclic AMP when exposed to ISO or CT unless the phosphodiesterase inhibitor MIX also was present (13).[3] Our present studies demonstrate that the desensitization phenomenon was very similar in both C6HP and C6LP cells. In addition, the time course of desensitization in C6HP cells was essentially the same whether or not the cells were exposed to MIX during the treatment with agonist. Furthermore, MIX by itself elevated cyclic AMP levels more than did ISO by itself; however, a 3-hr exposure to the former agent did not desensitize C6HP cells, whereas a similar treatment with ISO did. These results indicate that catecholamine-mediated desensitization of adenylate cyclase in rat glioma C6HP cells does not depend on cyclic AMP accumulation. Although we cannot rule out completely the possibility that a critical pool of cyclic AMP whose level is only modulated by *beta*-adrenergic agonists may have an effect on desensitization in C6HP cells, the observation that CT, which is effective as ISO in stimulating adenylate cyclase, does not cause the cells to become refractory to ISO would argue against this possibility.

Furthermore, C6HP cells, after being desensitized by ISO, remained completely responsive to CT. Experiments with C6LP cells were more complicated, as the ISO-treated cells appeared to be refractory to CT but less so when exposed to the toxin in the presence of MIX. Since adenylate cyclase of ISO-treated C6LP as well as C6HP cells was activated by CT either in intact cells or in membranes, these results suggest that the large accumulation of cyclic AMP during agonist treatment of C6LP cells may induce phosphodiesterase activity which masks the action of CT on the intact cells.[5]

Our conclusions are in contrast to those of Terasaki *et al.* (5), de Vellis and Brooker (10), and Nickols and Brooker (11, 12). These investigators have proposed that the desensitized (or refractory) state in rat glioma C6-2B cells is mediated by cyclic AMP, since ISO, MIX, and CT, agents that elevate cyclic AMP levels in these cells, cause the cells to become refractory to ISO. In addition, cells treated with ISO or with CT became refractory to the toxin. As these investigators did not directly measure adenylate cyclase activities, we cannot interpret their results in light of our own. However, Franklin and Twose (22) have recently reported that partial agonists cause desensitization in C6 cells without elevating cyclic AMP levels.

Nickols and Brooker (12) also have proposed that the refractory state in C6-2B cells is mediated by a "refractory protein" (the synthesis of which is induced by cyclic AMP) that affects the guanine nucleotide regulatory component of the adenylate cyclase system. This component, designated as G/F (23) or *N* (24, 25), is involved in the coupling of the *beta*-receptor to the catalytic component of adenylate cyclase and is the site at which guanine nucleotides, NaF, and CT mediate their effects on cyclase activity (23–28). However, we observed that ISO-stimulated adenylate cyclase activity was reduced in membranes from cells treated with the agonist whereas NaF-, guanine nucleotide-, and CT-stimulated activities were not reduced. No loss of NaF-stimulated activity was observed in membranes from ISO-treated C6-2B cells (5) or early passage C6 cells (21). Although C6LP as well as C6-2B cells accumulate very large amounts of cyclic

[5] Increases in phosphodiesterase activity in catecholamine-treated rat glioma C6 cells have been reported in some (10, 19, 20) but not other (21) studies. Induction of phosphodiesterase activity by catecholamines has not been observed in C6-2B cells (10).

AMP when exposed to *beta*-agonists, only a small rise in intracellular cyclic AMP is required to activate protein kinases and mediate subsequent cyclic AMP-dependent events (11, 22). Thus, even though the small increases in cyclic AMP levels observed in C6HP exposed to MIX should be sufficient to induce the synthesis of the purported refractory protein, this agent did not cause desensitization in C6HP cells.

Our results are more consistent with a model of catecholamine-induced desensitization which is specific and is mediated at the level of the receptor. Prolonged occupancy of the receptor by agonists results in an uncoupling of the receptor-adenylate cyclase complex without any observable alterations in other components of the complex. This latter conclusion is supported by the retention of NaF- and guanine nucleotide-stimulated activity in the desensitized cells. The ability of CT to activate adenylate cyclase in membranes from ISO-desensitized cells provides additional evidence that the guanine nucleotide regulatory protein which is ADP-ribosylated by the toxin (26–28) remains functional during desensitization. Its functionality has been implied but not directly demonstrated in previous studies (9). Thus, the mechanism of desensitization induced by *beta*-agonists in rat glioma C6 cells is similar to that observed in human astrocytoma cells (7–9), S49 mouse lymphoma cells (2, 9), human diploid fibroblasts (29)[6] and frog erthrocytes (30–31). Finally, we believe that the ability of CT to stimulate intact catecholamine-desensitized cells is highly significant, since it provides direct evidence that the above uncoupling model of desensitization is applicable to intact cells.[6] Effects of desensitization on the affinity of agonist binding to intact cells have not been reported and may not be possible to determine (31).

The uncoupling process may involve a modification of the receptor or an alteration in its membrane environment. The ability of membrane-active agents such as filipin (32–33), mellitin (6, 34), and phorbol esters (6, 35) to cause uncoupling of the *beta*-adrenergic-stimulated adenylate cyclase system (as well as other events related to cell differentiation) would support the latter mechanism. Phorbol esters and mellitin also are activators of phospholipase A_2 and it has been proposed that phospholipid turnover may be involved in the uncoupling process (6). Mepacrine, which inhibits phospholipase A_2, was shown previously to block ISO-mediated desensitization in rat glioma C6LP cells (6). We confirmed that observation but were unable to block desensitization in C6HP cells with mepacrine.[7] This observation indicates that additional differences exist between C6LP and C6HP cells in their catecholamine-stimulated adenylate cyclases. It also demonstrates that further studies are required to elucidate the molecular mechanism of the desensitization phenomenon.

[6] Human diploid fibroblasts desensitized to ISO remain responsive to CT as do guinea pig peritoneal macrophages (P. H. Fishman, unpublished observations). Thus, a functional regulatory component in intact catecholamine-desensitized cells appears to be a general phenomenon. Shear *et al.* (2) have reported that CT stimulated cyclic AMP accumulation in ISO-desensitized S49 cells, but did not discuss the implications of their observation.

[7] Mepacrine caused a 2-fold increase in the responsiveness of human fibroblasts to ISO, but did not block ISO-induced desensitization in these cells (P. H. Fishman, unpublished observations). Mepacrine, however, was able to block *beta*-adrenergic receptor desensitization *in vivo* as a result of ISO infusion or immobilization stress in rats (I. J. Kopin, personal communication to J. F. Tallman).

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