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Regulation of Adenylate Cyclase-Coupled *Beta* Adrenergic Receptor Binding Sites by *Beta* Adrenergic Catecholamines *in Vitro*

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SUMMARY

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Tolerance to catecholamines was studied using a frog erythrocyte model system *in vitro*. Incubation of cells with (−)-isoproterenol resulted in up to a 58% decline ($p < 0.001$) in maximum catecholamine-stimulated adenylate cyclase activity in membrane preparations. Desensitization was characterized by a decrease in V_{max} with no change in hormone affinity. Enzyme activity in the unstimulated state and after stimulation with NaF and prostaglandin E_1 (PGE_1) was unaltered. The number of functional *beta* adrenergic receptors, measured by binding of the *beta* adrenergic antagonist (−)-[^{3}H]dihydroalprenolol, showed a parallel decline of up to 47% ($p < 0.001$) with no change in receptor affinity. The dose-response curve for desensitization of enzyme activity and decreased receptor binding was the same as for (−)-isoproterenol stimulation of adenylate cyclase activity. The time course for the desensitization of adenylate cyclase and decrease in receptor number was much longer than that required for receptor binding or adenosine cyclic 3′,5′-monophosphate (cAMP) production, requiring 2–3 hr for completion. The ability of *beta* adrenergic agonists to cause a decrease in enzyme activity and in receptor number was directly related to their affinity for the *beta* adrenergic receptor. The potency series was isoproterenol > epinephrine > norepinephrine. The *beta* antagonist propranolol, while having no effect of its own, blocked the desensitization caused by isoproterenol. In contrast, the *alpha* adrenergic antagonist phentolamine did not interfere with the phenomenon. The desensitized state was shown to be reversible. If isoproterenol was removed from desensitized cells by washing, and cells were further incubated with propranolol, receptor binding and enzyme activity returned to nearly normal levels. Incubation of cells with PGE_1 produced desensitization to subsequent prostaglandin stimulation but had no effect on catecholamine sensitivity or *beta* receptor binding. Dibutyryl cAMP did not cause a decrease in binding or enzyme activity. It appears that catecholamines, through *beta* adrenergic receptor interactions, play a role in regulation of the number of functioning receptors at the cell surface. This may be a major mechanism for the induction of catecholamine tolerance.

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INTRODUCTION

Tolerance or desensitization to the effects of drugs or hormones is a common occurrence in clinical medicine and is frequently observed in many biological systems. The mechanisms of this adaptive reaction may vary with the type of drug, species of animal, and organ system. In any individual system, induction of metabolizing enzymes (1), depletion of neurotransmitters (2), increase in drug excretion (3), antidrug antibody production (4), or nonspecific target organ "fatigue" (5) might be involved. Recent evidence has shown that changes in the interaction of a drug or hormone with its specific receptor may play a role in the induction of some forms of desensitization (6–11). We have undertaken the study of catecholamine tolerance in which this newly recognized mechanism may play a significant role.

Tolerance to prolonged adrenergic stimulation occurs in humans and intact laboratory animals (12) and in isolated cells (13). At the cellular level, tolerance to the *beta* adrenergic effects of catecholamines has been associated with decreased responsiveness of adenylate cyclase to adrenergic stimulation, the first biological response that can be detected after exposure to hormone (14). We have chosen the frog erythrocyte as a source of *beta* adrenergic receptor-linked adenylate cyclase to study this phenomenon. Methods for measuring biological response to hormonal stimulation and for studying hormone and drug receptor interactions directly have been well characterized in this system (8, 10, 15–17). Binding of the radiolabeled *beta* adrenergic antagonist (−)-[^{3}H]dihydroalprenolol to membrane sites exhibits all the characteristics to be expected of ligand binding to the *beta* adrenergic receptor. In membrane fractions of canine heart (18) and frog erythrocytes (8, 19, 20), the binding shows ligand specificity, stereospecificity, affinity, and kinetics appropriate to the *beta* adrenergic receptor.

A preliminary communication from this laboratory (9) reported that isoproterenol-induced desensitization of adenylate cyclase in the frog erythrocyte system *in vitro* is accompanied by a decrease in the number of functional *beta* adrenergic receptors. We now describe detailed studies of catecholamine desensitization in this system *in vitro*. These studies reveal a striking correlation between the phenomena of desensitization and decline in number of *beta* adrenergic receptors under a wide variety of experimental conditions. These findings thus support the hypothesis that a reduction in receptor binding is a major mechanism in the induction of desensitization of tissues to catecholamines. The data also indicate that both the desensitization and decrease in receptor number are themselves *beta* adrenergic receptor-mediated events.

MATERIALS AND METHODS

(−)-[^{3}H]Dihydroalprenolol (specific activity, 17–33 Ci/mmole) was prepared by New England Nuclear Corporation by catalytic reduction of (−)-alprenolol (Hassle) with tritium gas, using palladium as a catalyst (8, 19). The compound was stored in absolute ethanol at −20°.

(−)-Alprenolol contains an unsaturated bond in the aliphatic chain on position 2 of the aromatic ring. We have recently demonstrated by mass spectroscopy that this material is, as anticipated, dihydroalprenolol. The material is homogeneous in six different chromatographic systems, as detailed elsewhere (20). In addition, the labeled material can be separated from native (−)-alprenolol by chromatography on silica gel plates impregnated with silver nitrate (20).

When the tritiated material is chromatographed alone on such silver nitrate-treated plates and the plates are examined by ultraviolet light, only a single spot is seen, corresponding to the R_F of the radioactivity. Thus no native, unreacted alprenolol contaminates the tritiated material.

The finding that the specific activity of the labeled material is less than "theoretical" (i.e., less than 60 Ci/mmole) is presumably due to tritium exchange with solvent during the labeling procedure, which leads to reduction of some double bonds with hydrogen rather than tritium atoms.

We have tested the biological activity of several lots of the labeled material, with specific radioactivities ranging from 10 to

33 Ci/mmole, as antagonists of isoproterenol-activated adenylate cyclase. In all cases the K_D obtained (about 3–5 nM) was the same and was in turn identical with that of unlabeled native (−)-alprenolol. Since the tritiated material contains no unreacted native (−)-alprenolol, these assays represent valid potency estimates of the tritiated compound (20).

Other drugs used in this study were (±)-propranolol HCl, (−)-isoproterenol, (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, cAMP,[2] ATP, phosphoenolpyruvate, and myokinase (all from Sigma). Phentolamine was obtained from Ciba; pyruvate kinase, from CalBiochem; and [^{3}H]cAMP (1–5 Ci/mmole) and [α-^{32}P]ATP (1–10 Ci/mmole), from New England Nuclear. Prostaglandin E_1 was a gift from Dr. John Pike, Upjohn Pharmaceuticals. Alumina, neutral grade, was obtained from ICN; Dowex AG 50W-X4, from Bio-Rad; and grass frogs (*Rana pipiens*), from Nasco-Steinhilber.

Frogs were bled by cardiac puncture, and the heparinized blood was washed twice in buffered amphibian saline (109 mM NaCl and 9 mM Tris-HCl, pH 7.4). Pooled blood was initially incubated at 23° with test drugs in a medium containing 101 mM NaCl, 17 mM Tris, 10 mM dextrose, 0.2 mM sodium metabisulfite, penicillin (50 units/ml), and streptomycin (5 mg/ml) at pH 7.4. Antibiotics were added to retard bacterial growth, which would have led to cell lysis. The medium was renewed after 12 hr in 24-hr incubation experiments. Cells were shielded from light and kept in suspension by slow rotation. These initial incubations lasted for 3–10 hr unless otherwise indicated.

After the initial incubation, 1-ml volumes of cells were washed in 40 ml of amphibian saline four times. Cells were then brought to a 3-ml volume in washing buffer (129 mM NaCl, 20 mM Tris, 5 mM EDTA, and 2 mM dithiothreitol, pH 7.4) and lysed by quick freezing in an ethanol–Dry Ice bath. Then 40 ml of washing buffer were added, and the lysate was thawed at 9°. After centrifugation at 30,000 × g for 15 min, the pellet was resuspended by homogenization in cyclase buffer (75 mM Tris and 25 mM $MgCl_2$, pH 7.4) and recentrifuged at 2000 × g for 8 min over a cushion of cyclase buffer containing 50% sucrose. The supernatant fraction was collected, pelleted at 30,000 × g for 15 min, and washed twice with washing buffer. This procedure resulted in eight separate washings after incubation. The final membrane preparation consisted of the pellet resuspended in cyclase buffer. Protein was determined by the method of Lowry *et al.* (21).

Adenylate cyclase assays were performed as reported previously (22, 23). Final incubation mixtures contained Tris-HCl, 30 mM, pH 7.4; $MgCl_2$, 10 mM; cAMP, 0.1 mM; ATP, 1.5 mM; [α-^{32}P]ATP, 1–2 × 10^6 cpm; phosphoenolpyruvate, 5 mM; pyruvate kinase, 40 μg/ml; and myokinase, 20 μg/ml. Incubations were carried out in 50-μl volumes for 15 min at 37°. [^{32}P]cAMP was isolated by the method of Salomon *et al.* (24). [^{3}H]cAMP was used to monitor for analytical losses during the chromatography. Recovery was generally about 70%.

(−)-[^{3}H]Dihydroalprenolol binding studies were performed essentially as described earlier (8, 17, 20). Membrane preparations (1–2 mg of protein) were incubated with 2–60 nM (−)-[^{3}H]dihydroalprenolol for 10 min at 37° in the presence and absence of unlabeled (±)-propranolol, 10 μM. Duplicate samples were layered over 0.3 ml cyclase buffer and centrifuged for 2 min in a Beckman Microfuge 152. The pellet surface was washed once. Pellets were solubilized with 10% sodium dodecyl sulfate and 10 mM EDTA overnight prior to addition of a Triton-toluene-based fluor and counting in a liquid scintillation spectrometer. Nonspecific binding was defined as the radioactivity bound to membranes incubated in the presence of 10 μM (±)-propranolol and was generally approximately 25% of the binding observed in incubations performed without propranolol. Specific binding was defined as the amount of radioactivity in samples incubated with (−)-[^{3}H]dihydroalprenolol alone, minus nonspecific binding. All values for (−)-[^{3}H]dihydroalprenolol binding refer to specific binding.

[2] The abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; PGE_1, prostaglandin E_1.

As described elsewhere (20), binding as assessed by this centrifugal method was essentially identical with that determined by equilibrium dialysis. Thus binding as assessed by this simple centrifugal assay is a true reflection of equilibrium binding (20).

In some later experiments, the membrane preparation was altered so that the sucrose separation step was omitted and an additional wash was added. With these membrane preparations, separation of receptor-bound (−)-[^{3}H]dihydroalprenolol from free drug was accomplished by dilution in ice-cold cyclase buffer and filtration through Whatman GFC glass filters, followed by additional washing. Dissociation of bound label was negligible. This method gave results comparable to the centrifugal assay, but with less than 10% nonspecific binding.

RESULTS

Effect of isoproterenol preincubation on adenylate cyclase activity. Membrane preparations from cells "initially" incubated with 0.1 mM (−)-isoproterenol for more than 5 hr displayed only 42% of the maximum catecholamine-stimulated adenylate cyclase activity present in control membranes ($p < 0.001$, paired t-test in 10 experiments). A typical isoproterenol dose-response curve (Fig. 1) shows that this desensitization was characterized by a decrease in maximum enzyme velocity (V_{max}) in response to stimulation, with no shift in apparent affinity for the drug (concentrations causing half-maximal stimulation). Enzyme activity was not different in control compared with isoproterenol-incubated cells in the basal (unstimulated) state or after stimulation with NaF or PGE_1 ($p > 0.7$, $p > 0.1$, and $p > 0.5$, respectively, for 10 experiments). Basal enzyme activity in treated cells was 37 ± 12.3 pmoles of cAMP generated per milligram of protein per minute, and in control cells, 38 ± 14 pmoles/mg of protein per minute.

Effect of isoproterenol preincubation on (−)-[^{3}H]dihydroalprenolol binding. In addition to the decreased enzyme sensitivity to subsequent *beta* adrenergic stimulation, membranes from isoproterenol-treated cells

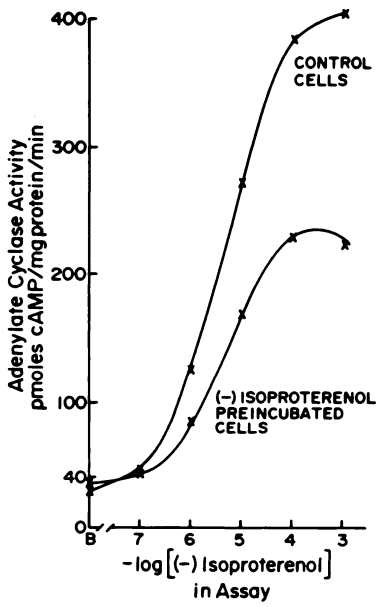

FIG. 1. *(−)-Isoproterenol stimulation of adenylate cyclase in frog erythrocyte membranes from cells preincubated with and without (−)-isoproterenol (10 μM)*

A typical experiment, determined in duplicate, is shown. B, basal, or unstimulated, enzyme activity.

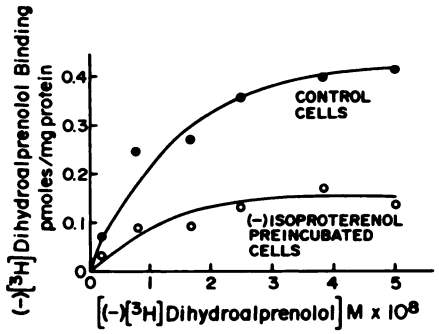

FIG. 2. *Specific (−)-[^{3}H]dihydroalprenolol binding in membranes from frog erythrocytes preincubated with and without (−)-isoproterenol (10 μM) as a function of (−)-[^{3}H]dihydroalprenolol concentration*

A typical experiment is shown. Each data point was determined in duplicate.

(0.1 mM, longer than 5 hr) showed a striking depression in binding of (−)-[^{3}H]dihydroalprenolol. Receptor binding in both treated and control preparations reached plateau at high (−)-[^{3}H]dihydroalprenolol concentrations, indicating that all available receptor sites were occupied, i.e., saturated (Fig. 2). This maximum

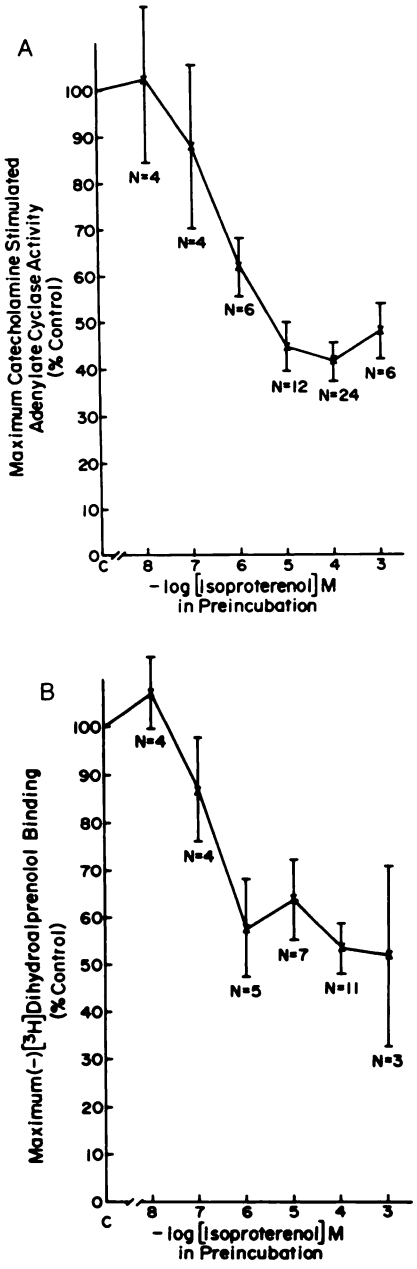

FIG. 3.

A. Maximum catecholamine-stimulated adenylate cyclase activity as a function of preincubation concentration of (−)-isoproterenol. Cells were incubated with 0.01–1000 μM (−)-isoproterenol for 5–10 hr as described in the text. Membrane fractions were assayed in duplicate for maximum adenylate cyclase activity in the presence of 0.1 mM (−)-isoproterenol. cAMP generation (picomoles per milligram of protein per minute) is expressed as a percentage of the control value for each experiment. N, number of experiments; C, control value. Brackets indicate standard errors of the mean.

B. Maximum (−)-[^{3}H]dihydroalprenolol binding as a function of preincubation concentration of (−)-isoproterenol. Incubation and assay conditions were the same as described in the text and previous figures. Binding was assayed in duplicate at two or three concentrations of (−)-[^{3}H]dihydroalprenolol above the saturation level (40–50 nM). Maximum binding refers to the average of these values, expressed as a percentage of the control value for each experiment. N, number of experiments; C, control value. Brackets indicate standard errors of the mean.

binding level corresponds to the number of functioning *beta* adrenergic receptors. In 13 experiments maximum binding in membranes from isoproterenol-desensitized cells was 53.2% of control ($p < 0.001$, paired t-test; isoproterenol concentration, 0.1 mM; longer than 5 hr). There was no change in the concentration of (−)-[^{3}H]dihydroalprenolol required to occupy half the receptors, a measure of receptor affinity for ligand.

Desensitization as a function of (−)-isoproterenol concentrations in initial incubation. Desensitization of adenylate cyclase and a corresponding decrease in the number of functioning *beta* adrenergic receptors were found to be dose-related phenomena. As shown in Fig. 3, there was a threshold to both effects at about 0.1 μM and a maximum at 10–100 μM (−)-isoproterenol. The concentration of (−)-isoproterenol causing half-maximal effects was between 0.1 and 1.0 μM for both phenomena. As noted above, the magnitude of enzyme desensitization (58% fall) also closely paralleled the reduction in *beta* receptor binding (47% decline).

Desensitization as a function of duration of initial incubation. The time course of desensitization of adenylate cyclase was nearly identical with that for the decrease in (−)-[^{3}H]dihydroalprenolol binding (Fig. 4). Decrements in binding and in adenylate cyclase activity were minimal before 30 min, nearly complete at 2 hr, and unchanged between 3 and 24 hr. All initial incubations were conducted with 0.1 mM (−)-isoproterenol.

Beta adrenergic specificity of desensitization to catecholamines. Figure 5 demon-

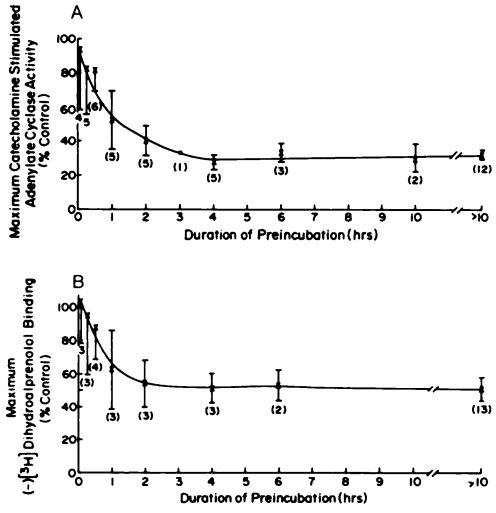

FIG. 4.

A. Maximum catecholamine-stimulated adenylate cyclase activity as a function of duration of preincubation with (−)-isoproterenol (0.1 mM). Incubations and assays were the same as described in the text. The duration of initial preincubation was measured from the addition of (−)-isoproterenol to the onset of washing. Four cell washes and freezing of cells were completed in less than 10 min. The number of experiments appears in parentheses beneath each data point. Brackets indicate standard errors of the mean.

B. Maximum (−)-[^{3}H]dihydroalprenolol binding as a function of duration of preincubation with (−)-isoproterenol. Incubations and assays were the same as described in the text and previous figures. The number of experiments appears in parentheses below each data point. Brackets indicate standard errors of the mean.

strates that a *beta* adrenergic agonist other than (−)-isoproterenol was capable of producing desensitization. Epinephrine, but not norepinephrine (at 10 μM), caused a reduction in adenylate cyclase sensitivity with a parallel drop in receptor binding. Epinephrine appeared less potent than (−)-isoproterenol at 10 μM.

Incubation of cells with the *beta* adrenergic antagonist propranolol had no effect on subsequent receptor binding or enzyme activity. However, as shown in Fig. 6, 10 μM propranolol effectively blocked the desensitization caused by 10 μM (−)-isoproterenol. Phentolamine, an *alpha* adrenergic antagonist shown previously not to interact with the *beta* adrenergic receptors in these preparations (8), had no effect by itself and did not block desensitization to (−)-isoproterenol. Unexplained is the apparent supersensitivity of adenylate cyclase after simultaneous incubation with (−)-isoproterenol and propranolol. Binding studies did not reflect this increased enzyme activity.

Resensitization. After cells had been incubated with 10 μM (−)-isoproterenol for 4 hr, enzyme desensitization and decline in *beta* adrenergic receptor number occurred as expected (Fig. 7). These cells were then washed three times, and the incubations were continued in the presence of 10 μM propranolol to ensure that no residual isoproterenol remained on the receptors. Eight hours later membranes were prepared and tested for catecholamine-sensi-

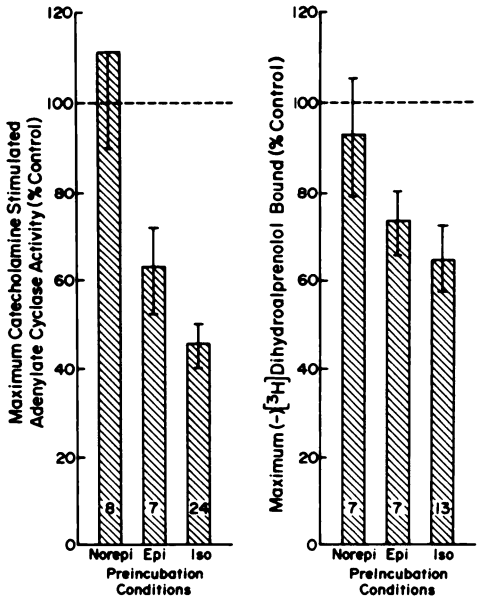

FIG. 5.

Left: Maximum catecholamine-stimulated adenylate cyclase activity as a function of preincubation with various agonists. Incubations and assays were the same as described in the text and previous figures. (−)-Isoproterenol (Iso), (−)-epinephrine (Epi), and (−)-norepinephrine (Norepi) were present at 10 μM in preincubations. The number of experiments appears inside the bars. Brackets indicate standard errors of the mean.

Right: Maximum (−)-[^{3}H]dihydroalprenolol binding as a function of preincubation with various agonists. Incubations and assays were the same as described in the text and previous figures. All agonists were present at 10 μM.

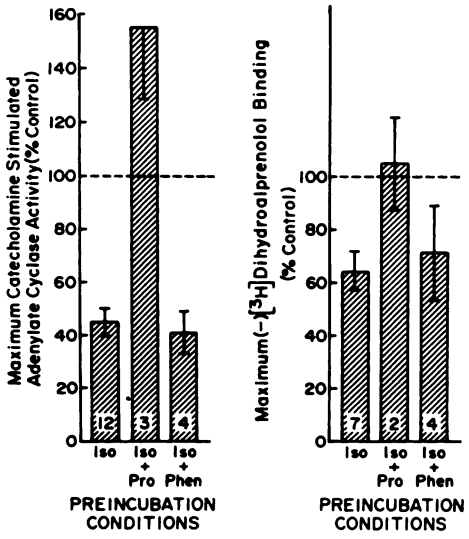

FIG. 6.

Left: Maximum catecholamine-stimulated adenylate cyclase activity after preincubation with (−)-isoproterenol and antagonists. Incubations and assays were the same as described earlier. The preincubation concentration of (−)-isoproterenol (Iso) was 100 μM in one experiment and 10 μM in all others. Both (±)-propranolol (Pro) and phentolamine (Phen) were present at 10 μM. The number of experiments appears inside the bars. Brackets indicate standard errors of the mean.

Right: Maximum (−)-[^{3}H]dihydroalprenolol binding after preincubation with (−)-isoproterenol and antagonists. Incubation conditions were the same as described for the left-hand graph.

tive adenylate cyclase and *beta* adrenergic receptor binding. During this 8-hr period the number of functioning receptors and the sensitivity of adenylate cyclase returned to nearly normal levels (Fig. 7). The increase in receptor number and in enzyme sensitivity was significant at the $p < 0.001$ level.

Induction of specific desensitization to PGE$_1$. Incubation of cells with PGE$_1$ (50 μM) resulted in a 60% decrease in the subsequent response of adenylate cyclase to prostaglandin stimulation ($p < 0.01$, $n = 4$). There was no desensitization to subsequent (−)-isoproterenol stimulation or decrement in (−)-[^{3}H]dihydroalprenolol binding. Incubation of cells with (−)-isoproterenol (10 μM) similarly resulted in no cross-tolerance to PGE$_1$. Subsequent stimulation of membranes from (−)-isoproterenol-treated cells by PGE$_1$ was no different from the control ($p > 0.5$, 10 experiments, paired t-test). These results are summarized in Table 1.

DISCUSSION

In many biological systems in which cAMP acts as a "second messenger," responsiveness to hormonal stimulation becomes attenuated with time; that is, tolerance or desensitization develops. In the frog red cell model, we have shown that this desensitization is characterized by up to a 58% fall in maximum *beta* adrenergic stimulated adenylate cyclase activity. Receptor affinity for hormone, assessed by dose-response curves, was not altered by desensitization. Advances in *beta* adrenergic receptor binding techniques enabled us to examine the changes in receptor binding characteristics that occurred as tissues became desensitized to catecholamines. These studies revealed a maximum decrement of 47% in the number of functionally active *beta* adrenergic receptor binding sites after adrenergic tolerance was induced, with no change in the binding affinity of the receptors.

That this correlation between decreased number of functioning receptors and decreased enzyme responsiveness is meaningful and not artifactually produced [by residual (−)-isoproterenol remaining after initial incubation or by a generalized toxic effect on the enzyme itself] is supported by the following data. First, basal enzyme activity in treated membranes was no higher than in control membranes, as would be expected if residual drug remained on the receptor. Second, addition of propranolol (10μM) to the enzyme assay did not significantly lower basal activity in treated cells, as would be observed if (−)-isoproterenol had not been washed off. Third, when cells were exposed to the same concentration of (−)-isoproterenol only briefly (less than 5 min) prior to washing, no desensitization resulted. Fourth, persistence of (−)-isoproterenol or a metabolite on the receptors would be expected to alter the apparent affinity of (−)-[^{3}H]dihydroalprenolol binding (shift the saturation curve to the right) without lowering the number of sites (maximum

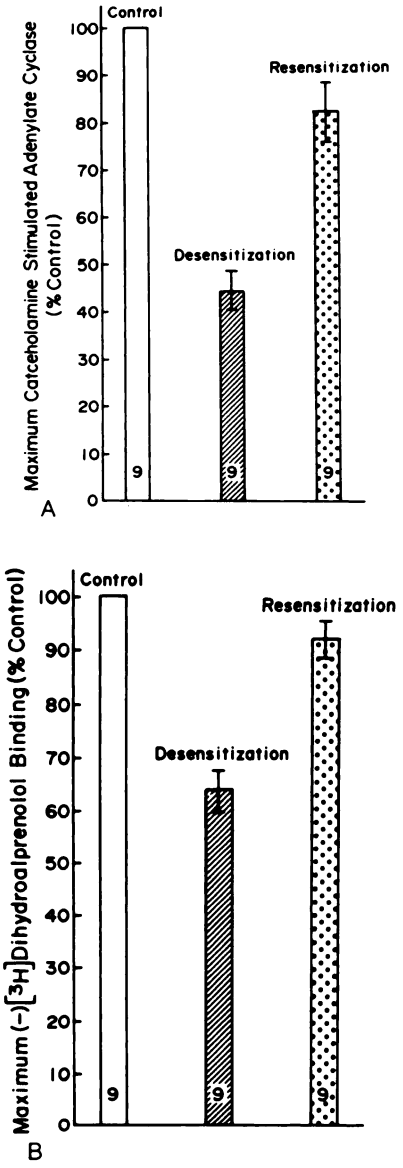

FIG. 7.

A. Resensitization of adenylate cyclase. Cells were incubated with 10 μM (−)-isoproterenol for 4 hr, and a portion was prepared for adenylate cyclase stimulation assay as described in the text and previous figures. A portion of the desensitized cells was then washed three times, incubated for an additional 8 hr with 10 μM propranolol (resensitization), and assayed as before. The number of experiments appears inside the bars. Brackets indicate standard errors of the mean.

B. Restoration of (−)-[^{3}H]dihydroalprenolol binding. The experimental protocol was the same as described for Fig. 7A. Assays were performed as described in the text and previous figures.

binding). In separate experiments we have demonstrated that this was precisely the result obtained when (−)-isoproterenol (0.5–5.0 μM) was actually added to control membranes prior to (−)-[^{3}H]dihydroalprenolol binding studies (11). Finally, basal enzyme activity as well as that after sodium fluoride and PGE_1 stimulation was unaltered, indicating that (−)-isoproterenol incubation did not have a toxic effect on the adenylate cyclase system itself. For these reasons, we are confident that our measurements of changes in receptor binding and enzyme activity are valid.

Recognizing that a loss of *beta* adrenergic receptor capacity could account for a major part of the observed adenylate cyclase desensitization, we conducted parallel studies of enzyme activity and receptor binding to characterize the effects more fully. The results of several experiments suggest that the first step necessary for the induction of desensitization is "occupation" of the receptors by a ligand. Two *beta* adrenergic agonists were capable of inducing tolerance in proportion to their *beta* adrenergic potency. Isoproterenol appeared to be more potent than epinephrine in producing desensitization at a concentration of 10 μM. The activities of (−)-isoproterenol in this system, as measured by receptor binding, by stimulation of adenylate cyclase, and by induction of desensitization, all follow the same dose-response relationships. All phenomena have a threshold at 0.1 μM, are half-maximal by 1 μM, and reach a plateau at 10–100 μM (−)-isoproterenol. These data are all consistent with the hypothesis that receptor occupancy is the first step in desensitization.

Although occupancy of the receptor seems to be necessary for induction of desensitization, ligand binding alone is clearly not sufficient. Incubation with the *beta* adrenergic antagonist propranolol at a concentration (10 μM) that fully saturates the binding sites resulted in no desensitization, suggesting that agonists alone possess a characteristic necessary for the production of desensitization. This hypothesis was tested by incubating cells with (−)-isoproterenol and (±)-propranolol together. The affinity of propranolol for the *beta* adrenergic receptors is 100 times

TABLE 1

Specific desensitization induced by (−)-isoproterenol and PGE_1

Values are the means and standard errors of the number of experiments shown in parentheses, each determined in duplicate. Unless otherwise indicated, values were not significantly different from controls ($p > 0.25$, paired t-test).

Initial incubation conditions	Maximum adenylate cyclase activity stimulated by:		Maximum [^{3}H]dihydroalprenolol binding
	Isoproterenol	PGE_1	
	% control		*% control*
PGE_1, 50 μM	108 ± 7 (4)	40 ± 6[a] (4)	122 ± 14 (4)
(−)-Isoproterenol, 100 μM	42 ± 4[b] (24)	88 ± 7 (11)	53 ± 6[b] (13)

[a] $p < 0.01$.
[b] $p < 0.001$.

that of isoproterenol (8), and at 10 μM concentrations the antagonist should effectively exclude the agonist from access to the *beta* receptor binding sites. Figure 6 shows that propranolol does in fact block (−)-isoproterenol-induced tolerance. Furthermore, the *alpha* antagonist phentolamine does not block either access of isoproterenol to receptors (8) or isoproterenol-induced catecholamine tolerance. In addition to demonstrating that receptor binding by agonist is necessary for desensitization, propranolol blockade of isoproterenol tolerance is significant additional evidence for the *beta* adrenergic mediation of this phenomenon.

The classification of an agent as an agonist or antagonist depends on its ability to evoke or to block a biological response—in this system, cAMP production. Since the primary difference between the *beta* adrenergic agents which did produce desensitization (agonists) and the one which did not (the antagonist propranolol) is the ability to stimulate adenylate cyclase, it seemed reasonable that the second messenger cAMP might be involved in producing desensitization. Several observations argue against this hypothesis, however. First, as shown in Table 1, incubation of cells with 50 μM PGE_1, which raises the level of intracellular cAMP[3] in this system, resulted in no change in (−)-[^{3}H]dihydroalprenolol binding or in enzyme sensitivity to catecholamines. Second, incubation of cells with dibutyryl cAMP (500 μM) resulted in no desensitization. Furthermore, specific desensitization could be induced to either PGE_1 or to catecholamines with no demonstrable cross-tolerance. Since both these agents stimulate increased intracellular cAMP levels, it seems unlikely that this cyclic nucleotide is involved in the desensitization to either hormone. Obviously these data do not rule out the role of cAMP, for arguments can be made for intracellular exclusion of endogenous and exogenous cyclic nucleotide from a possible necessary site of action.

Although it seems unlikely that desensitization depends on cAMP production, the time course data in Fig. 4 imply that events subsequent to receptor binding are required before tolerance can occur. Binding to the *beta* adrenergic receptor in frog erythrocyte membrane preparations reaches equilibrium within a few minutes, and is more than 80% complete in less than 1 min (8). Similarly, stimulation of adenylate cyclase by isoproterenol, an event subsequent to binding, has been shown to be nearly instantaneous (8). Desensitization, however, required 2–3 hr for completion at 0.1 mM isoproterenol, and preliminary data suggest that more time may be required at lower concentrations. Return of normal receptor binding and enzyme activity in desensitized cells also took hours for completion. For the described desensitization to be part of a physiological feedback control mechanism, it would be expected to be reversible. The experiments presented here demonstrate this characteristic, and are in agreement with those of Franklin *et al.* (25), who found recovery of *beta* adrenergic sensitivity to be a slow

[3] M. G. Caron and R. J. Lefkowitz, unpublished observations.

process which is delayed by low concentrations of isoproterenol.

Although the mechanisms through which hormone receptors become "inactivated" remain conjectural, there are some interesting relevant observations. Other workers in our laboratory, using an analogous frog erythrocyte model system *in vivo*, have shown that inhibition of protein synthesis with cycloheximide does not influence the enzyme or receptor changes of desensitization or recovery (11). Cooperative interactions among receptors in this system are equally influenced by agonists and antagonists (26, 27, 28), and therefore are also unlikely to be involved in desensitization. The totally *in vitro* nature of these experiments implies that no extra-erythrocyte influences (e.g., other hormones or organ systems) are necessary for either desensitization or resensitization. Although we favor the hypothesis of an agonist-induced conformational change in the *beta* adrenergic receptor, other mechanisms cannot be ruled out. Some proposed explanations include actual loss of receptors (destruction, release into the medium, internalization into the cell) and generation of relatively irreversibly bound inactive ligands (some unknown metabolic products of catecholamines).

It should be noted that although our studies strongly suggest that loss or inactivation of *beta* adrenergic receptor binding sites may be responsible for much of the observed adenylate cyclase desensitization, they do no prove that this is the only mechanism contributing to the desensitization. In fact, the observation that the decrease in catecholamine-stimulated enzyme activity was generally slightly greater than the decrease in number of receptors may indicate that other mechanisms are also operative. The results of these studies, obtained in a system entirely *in vitro*, directly parallel results of studies obtained by injecting catecholamine into living frogs (11). The close correspondence of results between the two frog erythrocyte systems, *in vivo* and *in vitro*, further validates our observations.

Catecholamine tolerance has been described in other model systems *in vitro*, including lymphoid tissues (29), cultured fibroblasts (25, 30), cultured pineals (31), human astrocyte cells (32), fat (33), rabbit cerebellum slices (34), and rat glioma cultures (35). Desensitization was measured in terms of cAMP accumulation (25, 35), adenylate cyclase activity (13), or enzyme induction (31). The only other report that characterizes enzyme changes in terms of enzyme velocity and hormone affinity, that of Franklin *et al.* (25), is in total agreement with our observations. However, no previous report has offered direct studies of the mechanism for these effects or explored changes in the hormone receptors themselves.

Reduction in functional receptor populations may be involved in other receptor-mediated processes. Studies *in vivo* and *in vitro* with insulin (6, 36, 37), acetylcholine (7, 38), and thyrotropin-releasing hormone (39) show that receptor binding is decreased by chronic hormone exposure. However, these reports do not offer parallel studies of both receptor binding and subsequent biological response.

Patients with markedly elevated levels of endogenous hormones [thyrocalcitonin (40), gonadotropins (41), or insulin (42)] have been shown to exhibit tolerance to the effects of these bioactive materials.

Feedback adjustment of the number of functional hormone receptors may play a major role in the maintenance of homeostasis in the face of changing levels of circulating hormones.

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