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Regulation of *Beta* Adrenergic Receptors in Isolated Frog Erythrocyte Plasma Membranes

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SUMMARY

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The ability of *beta* adrenergic agonists to inactivate reversibly the *beta* adrenergic receptor binding sites in isolated frog erythrocyte plasma membranes has been studied. When membranes were exposed to isoproterenol at 25°, 40-70% of the *beta* adrenergic receptors were rapidly lost, i.e., could no longer be assayed with (−)-[^{3}H]dihydroalprenolol. Although the rate of this process was considerably more rapid in membranes than previously found in whole cells, it was still slower than the rate of stimulation of the membrane-bound adenylate cyclase by isoproterenol. In agreement with previous findings in whole cells, the decreased receptor binding in membranes was associated with a fall in receptor number with no significant change in affinity of the remaining receptors. The specificity of this process in membranes was that of a *beta* adrenergic receptor-mediated effect. The order of potency of agonists in inducing the apparent fall in receptor number was (−)-isoproterenol> (−)-epinephrine>> (−)-norepinephrine. (−)-Isoproterenol was 1000 times more potent than (+)-isoproterenol. *Beta* adrenergic antagonists competitively antagonized the agonist-induced effect. (−)-Propranolol was 100 times more potent than (+)-propranolol in this regard. Among a series of 11 *beta* adrenergic agents tested, the ability to decrease receptor number maximally was directly correlated with maximum ability to stimulate adenylate cyclase (intrinsic activity); $r = 0.93$, $p < 0.001$. However, adenosine 3',5'-monophosphate (cAMP) did not appear to be involved in this desensitization process, since no substrate ATP was present during the incubations of membranes with isoproterenol, and cAMP at concentrations up to 0.5 mM did not reproduce the effect. Interventions which uncouple *beta* adrenergic receptors and adenylate cyclase, such as solubilization or filipin treatment of membranes, prevented the agonist-induced fall in receptor number. Guanine nucleotides and analogues, such as 5'-guanylylimidodiphosphate, completely prevented the agonist effect. Nucleotides also completely restored receptor number to normal after the agonist-induced lowering had occurred. Several sulfhydryl reagents, including *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, and dithiothreitol, markedly inhibited the agonist-induced fall in receptor number and also caused partial or complete restoration of receptor number toward normal. A wide variety of other group-specific reagents, as well as several oxidizing and reducing agents, were without effect. These data suggest the

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necessity for *beta* receptor-adenylate cyclase coupling as a primary requisite for the regulatory effect of agonists on the *beta* receptors. cAMP formation does not appear to be required.

INTRODUCTION

Beta adrenergic catecholamines, such as epinephrine and isoproterenol, often affect physiological processes by stimulation of the membrane-bound enzyme adenylate cyclase with consequent elevation of intracellular cAMP[2] levels (1). It has been demonstrated that exposure of several cell types to *beta* adrenergic agonists for minutes to hours leads to progressive, selective desensitization of adenylate cyclase to subsequent catecholamine stimulation (2–14). We have previously studied this phenomenon in a simple frog erythrocyte model system. When these cells are exposed to isoproterenol or other catecholamines *in vivo* (11, 13) or *in vitro* (12, 14), the membrane-bound adenylate cyclase becomes selectively desensitized to further *beta* adrenergic stimulation. Basal and fluoride- and prostaglandin E_1-sensitive enzyme activities are unaffected. The desensitization is accompanied by up to a 50–60% fall in the number of *beta* adrenergic receptors in the cell membranes, as assessed by binding studies with the potent *beta* adrenergic antagonist (−)-[^{3}H]dihydroalprenolol. Resensitization of the hormone response in association with a return in receptor number to normal occurs over a period of several hours when cells are washed free of catecholamines (13, 14). Inhibition of protein synthesis with cycloheximide does not retard resensitization or restoration of receptor number, suggesting that new protein synthesis is not required to "regenerate" the receptors (13).

In order to probe the molecular mechanisms involved in receptor regulation by *beta* adrenergic catecholamines, we have developed a cell-free system in which these phenomena can be investigated. Exposure of purified frog erythrocyte plasma membranes to *beta* adrenergic agonists leads to rapid, selective desensitization of the adenylate cyclase to catecholamine stimulation (15). This is accompanied by a fall in the number of *beta* adrenergic receptor binding sites in the membranes (15). In this communication we present detailed studies intended to evaluate possible mechanisms by which *beta* agonists regulate the properties of their receptors in these membranes. The data suggest a possible role for adenylate cyclase, unrelated to the generation of cAMP, in this process.

[2] The abbreviations used are: cAMP, adenosine cyclic 3′,5′-monophosphate; Gpp(NH)p, 5′-guanylylimidodiphosphate; PGE_1, prostaglandin E_1; NEM, *N*-ethylmaleimide; PHMB, *p*-hydroxymercuribenzoate; DTT, dithiothreitol.

MATERIALS AND METHODS

Materials. AMP, ATP, GTP, GDP, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, *N*-acetylimidazole, iodoacetamide, phenylmethylsulfonyl fluoride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and succinic acid were obtained from Sigma; digitonin and 2-mercaptoethanol, from Fisher; dithiothreitol, from Cyclo; L-lysine, from Calbiochem; Sephadex G-50, from Pharmacia; and Gpp(NH)p, from ICN. Southern grass frogs (*Rana pipiens*) were obtained from Nasco-Steinhilber.

(−)-Alprenolol was tritiated at New England Nuclear by catalytic reduction with tritium gas, using palladium as the catalyst, to a specific activity of 33 Ci/mmole. The tritiated material has the structure of (−)-[^{3}H]dihydroalprenolol and is chromatographically pure, as documented elsewhere (16). We have also previously shown the biological equivalence of native alprenolol and dihydroalprenolol as *beta* adrenergic antagonists (16, 17).

Unlabeled (−)-dihydroalprenolol used to dilute the (−)-[^{3}H]dihydroalprenolol for some binding studies was prepared at New England Nuclear by catalytic reduction of (−)-alprenolol with hydrogen. Its structure was authenticated by mass spectroscopy.

The sources of all other materials used have been previously reported (16).

Membrane preparations. "Purified" frog erythrocyte membranes were prepared as previously described (11, 15). Elsewhere we have documented that the specific activity of (−)-[^{3}H]dihydroalprenolol binding and adenylate cyclase are enhanced 5–10-fold in these preparations as compared with crude erythrocyte lysates (18).

Preliminary incubation of beta adrenergic receptors in membranes with agonists and antagonists. The membranes, suspended in 75 mM Tris-HCl, (pH 7.6) and 10 mM $MgCl_2$, were incubated with slow shaking for 30–60 min at 25° in the presence of different *beta* adrenergic agonists and antagonists (0.001–100 μM). Controls were incubated under identical conditions without added drugs. Incubations were generally performed in a volume of 1 ml containing 3–4 mg of membrane protein. At the end of incubations the membranes were diluted to 40 ml of 50 mM Tris-HCl (pH 7.6) and 10 mM $MgCl_2$ and centrifuged at 30,000 × *g* for 15 min. The membranes were washed two more times in the same way prior to their use for (−)-[^{3}H]dihydroalprenolol binding assays.

The washing procedures were conducted at 4° and generally consumed about 45 min. In separate experiments we have demonstrated that these washing procedures are sufficient either to remove or at least to reduce to biologically ineffective levels the concentrations of catecholamines, prostaglandins, or nucleotides used in these studies. Thus, when 100 μM isoproterenol or 10 μM PGE_1 is added to membranes which are then subjected to these washing procedures, adenylate cyclase activity returns to control levels after the wash. Similarly, the effect of GTP or Gpp(NH)p at 100 μM on *beta* receptor binding affinities (19) is completely reversed by these washing procedures. With regard to the effectiveness of these washing procedures in removing high-affinity compounds, it is shown below that propranolol, an agent with a 100-fold higher affinity for the *beta* receptors than isoproterenol, caused no detectable change in (−)-[^{3}H]dihydroalprenolol binding to membranes that had been incubated with concentrations as high as 100 μM. Mg^{2+} was routinely included in all buffers, since the purified frog erythrocyte membranes displayed a tendency to clump in its absence.

Inhibition of agonist-induced regulatory effect. Membranes were incubated at 25° for 45 min with and without 10 μM (−)-isoproterenol, together with different agents as described in the tables and figures. In the case of filipin-induced inhibition of desensitization, the membranes were incubated for 10 min at 25° with different concentrations of filipin (Fig. 5) before addition of 10 μM (−)-isoproterenol. At the end of the incubations the membranes were washed three times as described above and assayed.

Preliminary incubation of beta adrenergic receptors in whole cells. Pooled, washed cells were incubated at room temperature (25°) for 5 hr with and without 10 μM (−)-isoproterenol HCl as described previously (12, 14). The cells were then lysed, and purified membranes were prepared as described above and used for (−)-[^{3}H]dihydroalprenolol binding assays.

In figures and tables we use the term desensitization to refer to the process by which *beta* adrenergic agonists decrease the number of *beta* receptors in the membranes. Resensitization refers to a return in receptor number to normal.

In all experiments (−)-[^{3}H]dihydroalprenolol binding to control and isoproterenol-incubated membranes was compared by *t*-test; $p < 0.05$ was taken as a significant difference. Various reagents were tested for their effects on agonist-induced fall in receptor number or for ability to increase receptor number to normal after isoproterenol treatment. A reagent which prevented a significant fall in receptor number by isoproterenol was said to have prevented desensitization. Similarly, a reagent which increased receptor binding after isoproterenol treatment, such that the difference between control and treated membranes was no longer significant, was said to have resensitized the receptors.

Assay of (−)-[^{3}H]dihydroalprenolol binding in particulate preparations. Binding experiments were performed essentially as described previously (20, 21), using a glass fiber filtration method. The mem-

branes were assayed with (−)-[^{3}H]dihydroalprenolol at very high concentrations (about 0.2 μM) to assure that maximum receptor binding capacity was assessed. To achieve these high concentrations the radioligand was "diluted" with unlabeled material. In previous studies we have documented that saturation of *beta* adrenergic receptors in these membranes occurs at (−)-[^{3}H]dihydroalprenolol concentrations well below those used in these assays (11).

In all experiments the amount of (−)-[^{3}H]dihydroalprenolol nonspecifically bound to the membranes was determined by incubating membranes and (−)-[^{3}H]-dihydroalprenolol in the presence of 10 μM (−)-alprenolol. This nonspecific binding was subtracted from the total binding in all binding calculations. Specific binding was more than 80% of the total even at the very high radioligand concentrations used in these experiments.

Proteins. Proteins were determined by the method of Lowry *et al.* (22).

RESULTS

Time and temperature dependence of agonist-induced decrease in receptor number. When frog erythrocyte membranes were exposed to isoproterenol at 25°, the number of *beta* adrenergic receptors fell rapidly in a time- and concentration-dependent fashion. When incubation was performed at 4°, desensitization also occurred, but was less complete than at 25° (Fig. 1). These kinetic data appeared to be consistent with a second-order reaction (23). However, at the present level of experimentation we do not feel justified in attempting to calculate rate constants.

When isoproterenol-incubated membrane preparations were studied over a range of (−)-[^{3}H]dihydroalprenolol concentrations, it was found that the *beta* receptor number was lower (Fig. 2). Scatchard analysis (24) showed that there was no change in the binding affinity of the remaining receptors for (−)-[^{3}H]dihydroalprenolol (Fig. 2). This result agrees with previous findings when the receptors were desensitized in whole cells (11–14).

It was of interest that, as with results in whole cells, the agonist-induced decrease in receptors in membranes was only 50–60% of the total receptor population even at high isoproterenol concentrations. When whole cells or membranes were initially exposed to isoproterenol and washed and then the membranes were re-exposed to isoproterenol for 1 hr, no further reduction in receptor population occurred (data not shown).

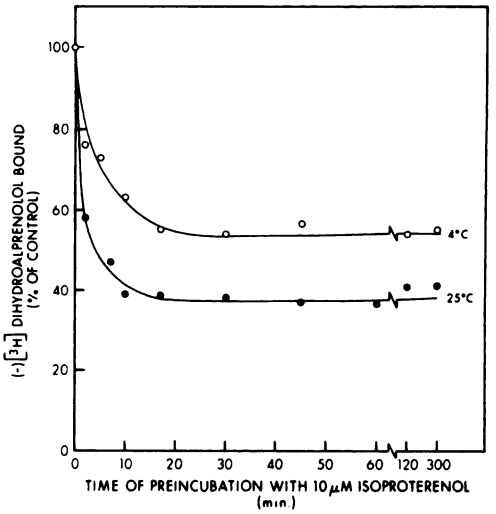

FIG. 1. *Temperature dependence of (−)-isoproterenol-induced reduction of (−)-[^{3}H]dihydroalprenolol binding sites in frog erythrocyte membranes*

The membranes were incubated with 10 μM (−)-isoproterenol at 4° or 25° for the indicated times. The desensitization reactions were stopped by adding 10 μM (±)-propranolol at the indicated times. Membranes were then washed as described under MATERIALS AND METHODS and assayed for (−)-[^{3}H]dihydroalprenolol binding. Control binding was 1.32 ± 0.16 pmoles/mg of protein. Results shown are the means of four experiments.

The agonist-induced decrease in receptor number appeared not to require divalent cations and was not inhibited by the chelator EDTA (data not shown).

Specificity of agonist-induced regulatory effect. The data in Table 1 indicate that ability to decrease receptor number during the 45-min preliminary incubation had the essential properties of a *beta* receptor-mediated process. The order of potency of agonists in decreasing receptor number was identical with that for occupancy of the receptors and stimulation of adenylate cyclase (16, 17). Cc_{34} and MJ 9184, however, seemed somewhat more po-

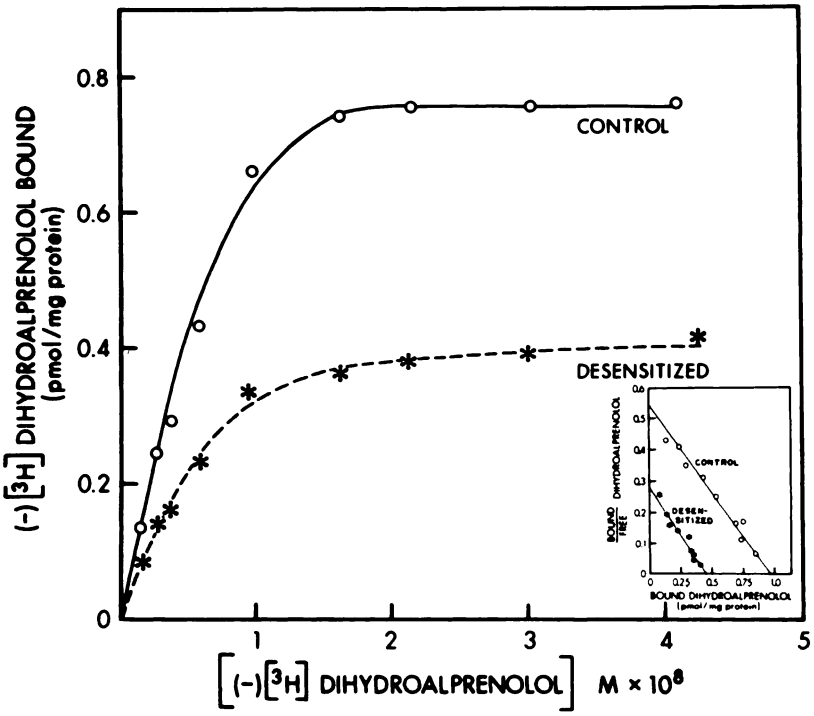

FIG. 2. *Specific (−)-[³H]dihydroalprenolol binding as a function of (−)-[³H]dihydroalprenolol concentration in control and desensitized frog erythrocyte membranes*

Desensitized membranes were treated with 10 μM isoproterenol for 45 min at 25°. Results shown are the mean of duplicate determinations from two experiments. Inset: Scatchard analysis (24) of (−)-[³H]dihydroalprenolol binding to control and desensitized membranes.

TABLE 1

Effects of beta adrenergic agonists on beta adrenergic receptor desensitization and adenylate cyclase stimulation

EC_{50} values are the concentrations causing half the maximal effect observed with the particular agent. Values for desensitization are taken from Fig. 4 and represent the means of four experiments. Values for adenylate cyclase activation are taken from a previous publication (16), except for salbutamol.

Agonist	EC_{50}	
	Desensitization	Adenylate cyclase activation
	μM	μM
(−)-Norepinephrine	200	150
(−)-Epinephrine	3	15
(−)-Isoproterenol	0.3	0.3
(+)-Isoproterenol	400	700
(−)-Soterenol	0.3	0.3
(±)-Salbutamol	0.5	1
(±)-Cc_{24}	0.007	0.08
(±)-MJ 9184	0.001	0.08

tent in desensitizing than in activating the receptors. As with other physiological effects of catecholamines, desensitization of the receptors was a stereospecific process. (+)-Isoproterenol was about 1000 times less potent than (−)-isoproterenol in decreasing receptor number.

At maximal concentrations the agonists did not lower receptor number to the same extent. Moreover, the extent to which any agent maximally reduced receptor number during the preliminary incubation was directly related to the ability of that agent to stimulate adenylate cyclase maximally. Thus soterenol and salbutamol, which are partial agonists for adenylate cyclase stimulation, only partially lowered receptor number even at maximally effective concentrations. This finding is in good agreement with recent findings of others that the partial agonist salbutamol only partially desensitizes the catecholamine-induced cAMP response of cultured fibroblasts as compared with the full agonist

isoproterenol (4). Cc_{34}, a potent agonist which has an intrinsic activity or efficacy even higher than isoproterenol (16), lowered receptor number to an even greater extent than isoproterenol. In contrast, antagonists such as (−)- and (+)-propranolol and dichloroisoproterenol, which do not stimulate the adenylate cyclase (intrinsic activity = 0), did not desensitize the receptors after incubation with membranes. A plot of intrinsic activity of several adrenergic agents determined by adenylate cyclase activation (16) against their intrinsic activity determined by ability to lower *beta* receptor number during a 45-min preliminary incubation is presented in Fig. 3. A straight-line relationship is obtained with a correlation coefficient of 0.93 and $p < 0.001$.

It should be borne in mind that although the ability of the various adrenergic agents to regulate receptor number was directly related to their ability to activate adenylate cyclase, this effect presumably did not result from cAMP production as a result of such enzyme activation, since there was no substrate ATP present for the enzyme during the initial incubations of the washed membranes in Tris-Mg^{2+} buffer. Second, when cAMP was added to the membranes at concentrations as high as 0.5 mM with or without 10 mM theophylline, it did not decrease receptor number.

Inasmuch as antagonists such as propranolol did not cause a fall in receptor number, their ability to prevent the effect of isoproterenol was tested (Fig. 4). *Beta* blockers, such as (−)-propranolol and (±)-dichloroisoproterenol, inhibited the isoproterenol-induced fall in receptor number. Furthermore, the inhibition was stereospecific. Thus (+)-propranolol also inhibited desensitization, but at 100-fold higher concentrations than (−)-propranolol. (+)-Propranolol and (±)-dichloroisoproterenol were essentially equipotent in inhibiting desensitization, which agrees with their equipotency in antagonizing *beta* receptor-mediated stimulation of adenylate cyclase in these membranes (16, 17). Phentolamine, an *alpha* adrenergic antagonist, did not inhibit desensitization at 10 μM. These results further support the contention that the receptor regulatory effect is itself a *beta* adrenergic receptor-mediated event.

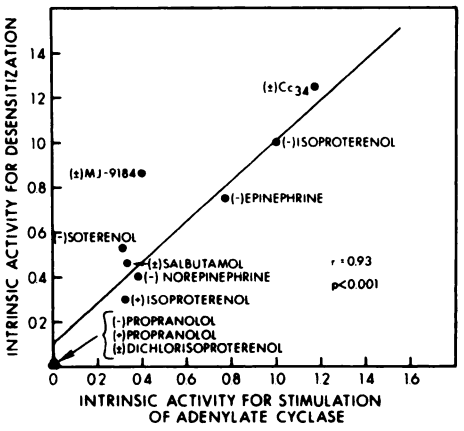

FIG. 3. *Correlation of intrinsic activity of beta adrenergic agents in desensitizing beta adrenergic receptors and activating adenylate cyclase*

Values for desensitization were calculated as the ratio of maximum desensitization induced by any agent to that induced by high concentrations of isoproterenol. Values for adenylate cyclase activation are taken from our previous work (16) and several additional experiments.

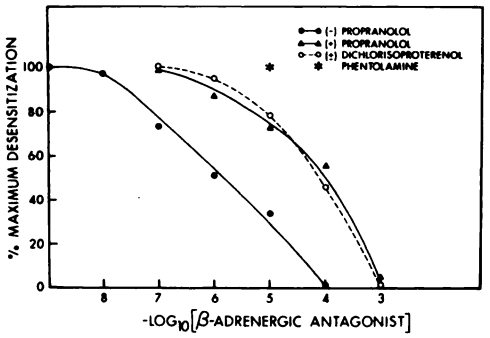

FIG. 4. *Inhibitory effects of beta adrenergic antagonists on (−)-isoproterenol-induced reduction of (−)-[^{3}H]dihydroalprenolol binding sites*

The membranes were incubated with 10 μM (−)-isoproterenol together with different concentrations of (−)- or (+)-propranolol, (±)-dichloroisoproterenol, or phentolamine at 25° for 45 min. Control membranes were incubated without added drugs. Membranes were washed as described in MATERIALS AND METHODS prior to binding assays. The results shown are the means of three experiments determined in duplicate. The maximum reduction in beta adrenergic receptor number was 60% of the total. Zero % maximum desensitization means that binding equivalent to the control value was observed.

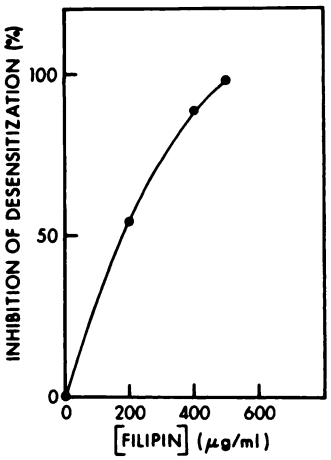

FIG. 5. *Effects of filipin on isoproterenol-induced desensitization of beta adrenergic receptors*

The membranes were treated with filipin for 10 min at 25° and then incubated with or without 10 μM isoproterenol for 45 min at 25°. The membranes were washed three times and assayed for (−)-[^{3}H]dihydroalprenolol binding. Values shown are the means of duplicate determinations from three to five separate experiments.

Effects of filipin. Filipin is a polyene antibiotic which has been shown to interact with the hydrophobic core of biological membranes (25). In amphibian (26) and possibly avian (27) erythrocyte membranes, one result of this interaction is an apparent uncoupling of *beta* adrenergic receptors and adenylate cyclase. Thus, in filipin-treated frog erythrocyte membranes, *beta* adrenergic receptor binding is essentially unaltered, whereas catecholamine-stimulated adenylate cyclase is selectively lost (26). Accordingly it seemed of interest to test the effects of filipin on the agonist-induced fall in receptor number (Fig. 5). Prior treatment of membranes with filipin caused dose-related inhibition of the isoproterenol-induced decrease in receptors. Thus the uncoupling of *beta* adrenergic receptors and adenylate cyclase caused by filipin treatment of the membranes is associated with an impaired ability of isoproterenol to regulate the *beta* adrenergic receptors.

Effects of nucleotides and nucleotide analogues. The presence of several purine nucleotides or nucleotide analogues during preliminary incubation of membranes with isoproterenol partially or completely prevented the regulatory effect of the agonists on the receptors. Gpp(NH)p was most active, but GTP and GDP were also effective, as was ATP, at millimolar concentrations (Table 2).

Prevention of the agonist effect by GTP or Gpp(NH)p required the continued presence of the nucleotide. When membranes were initially exposed to nucleotide, then washed and exposed to isoproterenol, reduction in receptor binding occurred in the usual fashion and to the same extent (Table 2).

A concentration curve for the Gpp(NH)p effect in preventing the agonist-induced fall in receptor number shows that the half-maximal effect of the nucleotide analogue occurred at 6 μM (Fig. 6). This is very comparable to the concentration of Gpp(NH)p (2 μM) which causes half-maximal stimulation of adenylate cyclase in these membranes (19), but considerably lower than the concentration of Gpp(NH)p (30 μM) previously found to cause half-maximal return in receptor number to-

TABLE 2

Inhibition of desensitization by nucleotides

Membranes were incubated at 25° for 45 min with and without 10 μM (−)-isoproterenol, with different nucleotides at the concentrations indicated, then washed three times before binding assays were performed. Results are the means and standard errors of duplicate determinations in four experiments. Differences between control and desensitized membranes were compared by the *t*-test.

Addition	(−)-[^{3}H]Dihydroalprenolol bound	
	Control	Desensitized
	pmoles/mg protein	
None	1.65 ± 0.29	0.84 ± 0.12[a]
Gpp(NH)p, 0.1 mM	1.71 ± 0.05	1.9 ± 0.04
Gpp(NH)p, 0.1 mM, then washed[b]	1.17 ± 0.14	0.5 ± 0.12[a]
GTP, 1 mM	1.7 ± 0.2	1.49 ± 0.03
GDP, 2 mM	1.75 ± 0.4	0.97 ± 0.25
ATP, 2 mM	1.53 ± 0.34	1.12 ± 0.1
cAMP, 0.5 mM	1.63 ± 0.24	0.8 ± 0.31[a]

[a] $p < 0.05$ compared with control membranes.

[b] These membranes were incubated at 25° for 10 min with Gpp(NH)p, then washed twice with buffer, before the desensitizing incubation with 10 μM (−)-isoproterenol.

ward normal after incubation of membranes with isoproterenol (15).

We have recently demonstrated that Gpp(NH)p and other guanine nucleotides also completely reverse the decrease in receptor number induced by incubating membranes with isoproterenol for 1 hr (15). A series of experiments was designed to test whether or not the desensitized state would remain readily reversible by nucleotides (Table 3). When membranes were exposed to isoproterenol at 25° or 4° for 5 hr, receptor inactivation remained fully reversible by Gpp(NH)p. When the number of *beta* adrenergic receptors was decreased by incubating whole cells with isoproterenol for 5 hr as described in previous studies (12, 14), and membranes from these cells were then exposed to Gpp(NH)p, the decrease in receptor number was found to be less readily reversible. Thus, under these conditions, a statistically significant difference between control and isoproterenol-treated membranes persisted (Table 3) after Gpp(NH)p treatment. Additional processes may be operative in intact cells which, with time, convert the "inactivated" receptors from a state which is readily reversible by nucleotides to one which is not.

When membranes were exposed to isoproterenol and then solubilized with digi-

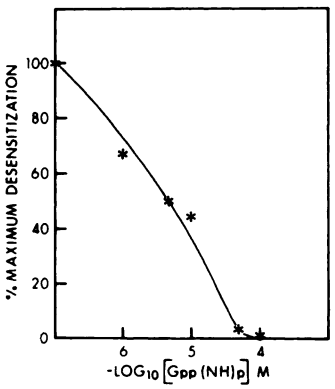

FIG. 6. *Inhibition of desensitization by Gpp(NH)p (NH)p*

Membranes were incubated with and without 10 μM (−)-isoproterenol, together with the indicated concentrations of Gpp(NH)p, for 45 min at 25°. Maximum desensitization refers to that observed in the absence of Gpp(NH)p. Zero % maximum desensitization means that no desensitization occurred. Results shown are means of duplicate determinations from two separate experiments.

TABLE 3

Effect of desensitization conditions on subsequent resensitization by Gpp(NH)p

Membranes or whole cells were desensitized with 10 μM (−)-isoproterenol for the indicated times at the temperatures shown. Controls were incubated under identical conditions, but without isoproterenol. The preparations were then washed as described under MATERIALS AND METHODS. When whole cells were incubated, membranes were prepared before the binding assays. Aliquots of control and desensitized membranes were assayed for (−)-[^{3}H]dihydroalprenolol binding; values are listed in the first two columns of results. The remainder of the control and desensitized membranes were incubated for 5–10 min at 25° with 0.1 mM Gpp(NH)p and then assayed for (−)-[^{3}H]dihydroalprenolol binding; values are listed in the last two columns. When whole cells were desensitized, resensitization was performed on washed membranes. Solubilized preparations were incubated with Gpp(NH)p after solubilization; these preparations were solubilized and assayed for (−)-[^{3}H]dihydroalprenolol binding as described previously (18). Values are the means and standard errors of duplicate determinations in the number of experiments indicated in parentheses. Differences with respect to controls were compared by the *t*-test.

Desensitization conditions	(−)-[^{3}H]Dihydroalprenolol bound			
	Control	Desensitized	Control	Resensitized
	pmoles/mg protein		*pmoles/mg protein*	
Membranes at 25°, 5 hr (4)	1.66 ± 0.42	0.52 ± 0.16[a]	1.82 ± 0.48	1.27 ± 0.2
Membranes at 4°, 5 hr (4)	1.02 ± 0.05	0.57 ± 0.03[a]	1.0 ± 0.06	1.02 ± 0.04
Whole cells at 25°, 5 hr (6)	1.1 ± 0.05	0.65 ± 0.04[b]	1.21 ± 0.11	0.87 ± 0.09[a]
Membranes at 25°, 1 hr, then solubilized with digitonin (4)	1.51 ± 0.12	0.32 ± 0.05[b]	1.41 ± 0.09	0.55 ± 0.04[b]

[a] $p < 0.05$ compared with control.
[b] $p < 0.001$ compared with control.

tonin, the decrease in receptor number persisted. When such solubilized preparations were exposed to Gpp(NH)p, the number of receptors did not increase (Table 3).

The Gpp(NH)p-resensitized state of the *beta* adrenergic receptors differs from their ground state. The binding affinity of nucleotide-resensitized receptors for agonists is decreased about 10-fold, whereas affinity for antagonists is unaltered. This observation is in agreement with findings for undesensitized receptors exposed to guanine nucleotides (19) and suggests that the nucleotide-altered state of the receptors reached from the ground state and the desensitized state is the same. When nucleotide is washed out of such preparations, binding affinities closely corresponding to the ground state are again observed (data not shown).

Effects of fluoride and PGE$_1$. In view of the striking effects of guanine nucleotides on the agonist-induced fall in receptor number, it seemed of interest to test the effects of other agents capable of stimulating the adenylate cyclase. In this system F^- stimulates the enzyme presumably via an effect on the catalytic unit, whereas PGE_1 stimulates it apparently through a distinct receptor mechanism. As shown in Table 4, NaF at 10 mM (a concentration which produces maximum effects on adenylate cyclase) completely prevented the isoproterenol-induced decrease in receptors. PGE_1 at 10 μM had no effect. These findings suggest that the conformational alterations in adenylate cyclase produced by NaF but not PGE_1 are sufficient to prevent subsequent *beta* receptor inactivation by the enzyme. By contrast, after the reduction in receptor number had occurred, NaF at 10 mM caused no increase in receptor number while PGE_1 may have caused slight resensitization (Table 4). Neither PGE_1 or NaF had any effect on control (−)-[^{3}H]dihydroalprenolol binding (data not shown).

Effects of sulfhydryl and other reagents. When NEM (2 mM), PHMB (0.5 mM), DTT (2 mM), or 2-mercaptoethanol (2 mM) was present during incubations of membranes with isoproterenol, the fall in receptor number was markedly inhibited (Table 5). Similarly, when membranes were incubated with isoproterenol, washed, and then exposed to these reagents, significant increases in receptor number occurred (Table 5). The effects of NEM were completely prevented by its prior reaction with an equivalent amount of 2-mercaptoethanol. As indicated in Table 5, a variety of other group-specific reagents appeared to be without effect. In addition to the sulfhydryl reagents, *N*-acetylimidazole, L-lysine, and carbodiimide also led to a loss of the significant difference between binding in control and desensitized membranes. However, inspection of the data suggests

TABLE 4

Effects of fluoride and PGE$_1$ *on desensitization and resensitization of beta adrenergic receptors*

For desensitization, the membranes were incubated at 25° for 45 min with and without 10 μM (−)-isoproterenol, in the presence and absence of 10 μM NaF or 10 μM PGE_1. Membranes were washed three times before assay. For resensitization, the washed control and desensitized membranes were incubated at 37° for 10 min with PGE_1 or NaF before assay. Values are the means and standard errors of the number of experiments shown. Differences with respect to controls were compared by the *t*-test.

Addition	(−)-[^{3}H]Dihydroalprenolol bound					
	Desensitization			Resensitization		
	Control	Desensitized	*n*	Control	Desensitized	*n*
	pmoles/mg protein			*pmoles/mg protein*		
None	1.07 ± 0.18	0.47 ± 0.08[a]	6	1.38 ± 0.2	0.71 ± 0.19[b]	8
PGE_1	1.4 ± 0.16	0.66 ± 0.05[c]	6	1.45 ± 0.35	0.93 ± 0.38	8
NaF	1.58 ± 0.14	1.61 ± 0.11	6	1.12 ± 0.18	0.5 ± 0.14[a]	6

[a] $p < 0.02$ compared with control.
[b] $p < 0.05$ compared with control.
[c] $p < 0.005$ compared with control.

TABLE 5

Effects of group-specific reagents on desensitization and resensitization of beta adrenergic receptors

For desensitization, the membranes were incubated at 25° for 1 hr with and without 10 μM (−)-isoproterenol and the various group-specific reagents, at the concentrations indicated. The membranes were then washed and assayed for (−)-[^{3}H]dihydroalprenolol binding. For resensitization, the washed control and desensitized membranes were incubated at 25° for 10 min with the various group-specific reagents before the binding assay. Values are the means and standard errors of the number of experiments shown. Differences with respect to controls were compared by the *t*-test.

Reagent	(−)-[^{3}H]Dihydroalprenolol bound					
	Desensitization			Resensitization		
	Control	Desensitized	*n*	Control	Desensitized	*n*
	pmoles/mg protein			*pmoles/mg protein*		
None	1.21 ± 0.14	0.62 ± 0.11[a]	7	1.38 ± 0.13	0.73 ± 0.08[a]	12
N-Ethylmaleimide, 2 mM	1.31 ± 0.23	1.32 ± 0.23	7	1.52 ± 0.16	1.40 ± 0.14	12
p-Hydroxymercuribenzoate, 0.5 mM	1.0 ± 0.24	0.79 ± 0.09	4	1.1 ± 0.18	1.13 ± 0.2	8
Dithiothreitol, 2 mM	1.44 ± 0.2	1.2 ± 0.29	6	1.17 ± 0.11	1.0 ± 0.1	10
2-Mercaptoethanol, 2 mM	1.42 ± 0.31	1.0 ± 0.13	4	1.82 ± 0.2	1.3 ± 0.05[b]	6
N-Acetylimidazole, 2 mM	1.31 ± 0.2	0.61 ± 0.09[b]	3	1.60 ± 0.36	0.9 ± 0.21	3
Iodoacetamide, 1 mM	1.46 ± 0.14	0.64 ± 0.06[b]	2	2.04 ± 0.16	0.78 ± 0.25[b]	2
Phenylmethylsulfonyl fluoride, 2 mM	1.26 ± 0.16	0.61 ± 0.1[b]	3	2.03 ± 0.24	0.98 ± 0.22[b]	3
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, 2 mM	1.28 ± 0.2	0.52 ± 0.14[b]	3	1.85 ± 0.31	1.00 ± 0.26	3
L-Lysine, 2 mM	1.34 ± 0.16	0.68 ± 0.14[b]	3	1.71 ± 0.34	0.92 ± 0.3	3
Succinate, 2 mM	1.48 ± 0.21	0.70 ± 0.26[b]	3	2.18 ± 0.41	0.93 ± 0.16[b]	3

[a] $p < 0.01$ compared with control.
[b] $p < 0.05$ compared with control.

that this may be due to the large standard error and limited number of these particular experiments.

The oxidizing agents H_2O_2 and potassium ferricyanide, as well as the reducing agent ascorbic acid, were tested at 1 mM concentrations for effects on agonist-induced decreases in receptor binding and resensitization. None of these agents altered the fall in receptor number induced by isoproterenol, and none caused any increase in receptor number after prior isoproterenol treatment. Similarly, these reagents were without effect on control binding of (−)-[^{3}H]dihydroalprenolol.

DISCUSSION

In the past several years a great deal of investigative effort has focused on the problem of hormone- and drug-induced desensitization. However, virtually all previous studies of these phenomena have been performed in whole cell preparations either *in vivo* or *in vitro*. Although such studies have been useful in establishing the major characteristics of these phenomena, they do not lend themselves to detailed biochemical investigations of possible mechanisms responsible for desensitization. By contrast, the purified plasma membranes used in these studies may be more suitable for such studies. The occurrence of regulation of the *beta* adrenergic receptors by *beta* adrenergic agonists in these isolated membranes essentially excludes a variety of processes as possible mediators of these events. Thus metabolic processes and protein synthesis cannot be involved. Reversible conformational alterations of the receptors seem a more likely molecular mechanism.

It should be noted however, that the characteristics of the regulatory process in these isolated membranes are somewhat different from those in intact cells. Thus, for example, the reduction in receptor number occurs more rapidly, and is more readily reversed by nucleotides. In addi-

tion, the lability of adenylate cyclase in the isolated membranes complicates an effective investigation of adenylate cyclase desensitization in this system.

Our current formulation of the possible mechanism of the agonist-induced fall in receptor number in this cell-free system is as follows. Perhaps the most important point is the necessity for receptors to interact with adenylate cyclase in order to become inactivated. Several lines of evidence suggest that this is so. First, only agonists (which presumably promote receptor-enzyme coupling) and not antagonists (which do not promote such coupling) cause the fall in receptor number. Moreover, among a series of partial agonists, the ability to decrease receptor number was directly related to ability to activate the adenylate cyclase. Second, interventions which functionally uncouple receptors and adenylate cyclase, such as solubilization (15) or filipin treatment, prevent or inhibit the regulatory effect. Despite the evidence that receptor–adenylate cyclase coupling is necessary, cAMP formation appears not to be involved, and cAMP itself does not produce a fall in receptor number.

The time course for the decrease in receptor number is slower than that for agonist-induced receptor-cyclase coupling (enzyme stimulation), which is known to be virtually instantaneous (28). The greatly accelerated time course of this process in the isolated membranes as opposed to intact cells (12, 14) may be a reflection of a loss of resensitizing factors (nucleotides?) in the membranes. Our data also suggest that a SH– → –S–S– transition may be involved in the conformational changes which occur in the receptors under the influence of agonists. Alternatively, the effects of the sulfhydryl reagents might be due entirely to effects on sulfhydryl groups in the adenylate cyclase.

Guanine nucleotides are capable both of increasing receptor number to normal after agonist-induced decreases and of preventing the agonist-induced fall in receptor number. The ability of Gpp(NH)p to prevent the effects of isoproterenol was concentration-dependent and occurred over the same concentration range as that for Gpp(NH)p stimulation of adenylate cyclase (19, 29). It is therefore possible that the ability of Gpp(NH)p and other nucleotides to influence the desensitization process may be mediated by effects of these agents on the cyclase enzyme, which are then in some way transmitted to the receptors. Elsewhere we have demonstrated that Gpp(NH)p and other guanine nucleotides selectively alter the binding affinity of agonists but not antagonists for the *beta* adrenergic receptors in frog erythrocyte membranes (19). When we examined the affinity of nucleotide-resensitized receptors for agonists, this was found to be selectively altered as well. Thus the nucleotide-altered conformation of the *beta* adrenergic receptors can be reached either directly, from the ground state of the receptors, or indirectly, from the desensitized state. The nucleotide-altered conformation reverts to the ground state when nucleotide is removed, for example, by washing of the membranes. This differs from the stimulation of adenylate cyclase induced by Gpp(NH)p, which persists after washing of membranes (30).

In our formulation agonist-induced coupling between *beta* receptors and adenylate cyclase is crucial for the decrease in receptor number. Since guanine nucleotides appear to enhance coupling in several systems, it might have been expected that GTP and Gpp(NH)p would promote rather than prevent desensitization. However, these nucleotides also appear to cause marked conformational alterations in the enzyme as well (29, 30), which may sufficiently alter the receptor-cyclase interaction so that the necessary conformational alterations in the receptors cannot occur. Similarly, the ability of fluoride to prevent the fall in receptor number is presumably related to its ability to stimulate the cyclase markedly (by conformational or other alterations).

One of the major implications of this formulation is the delineation of a function for the adenylate cyclase enzyme, independent of cAMP generation. This role is the regulation of the number of "functional" *beta* adrenergic receptors. Thus conventional schemes of hormone action,

which generally indicate unidirectional flow of information from receptor to enzyme, may need to be altered to take into account information flow in the opposite direction, i.e., regulation of receptors by the adenylate cyclase. These studies do not elucidate the nature of the conformational alterations in the *beta* receptors induced by agonists which render them inactive or "desensitized." One interesting speculation is that the agonists may induce a "high-affinity" state of the receptor which very tightly binds agonist but which is not functional in activating adenylate cyclase and also does not bind the ligand (−)-[^{3}H]dihydroalprenolol.

It should also be stressed that although these studies have focused on receptor alterations in relation to catecholamine desensitization, they in no way exclude other possible mechanisms. In fact, a variety of different mechanisms for desensitization and resensitization have been described in several systems. For example, in some systems hormones may regulate receptor concentrations by affecting the rates of synthesis or degradation (31, 32) of their own receptors. In another system, refractoriness induced in ovarian tissue by human chorionic gonadotropin may involve phosphorylation of some component of the system (receptor?) (33). In cultured human astrocytoma cells, cAMP itself is thought to be the feedback regulator which leads to desensitization (34). There is also evidence that hormonally mediated changes in phosphodiesterase activity may contribute to some forms of desensitization (35). Thus agonist-induced conformational alterations in receptors are only one of a number of potential mechanisms by which agonist drugs and hormones might regulate the responsiveness of their target tissues.

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