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Desensitization of β-adrenergic receptors by β-adrenergic agonists in a cell-free system: Resensitization by guanosine 5′-(β,γ-imino)triphosphate and other purine nucleotides

[(−)[^{3}H]dihydroalprenolol/binding sites/adenylate cyclase/catecholamines]

CHHABIRANI MUKHERJEE AND ROBERT J. LEFKOWITZ

Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT **Incubation of purified frog erythrocyte membranes with β-adrenergic agonists at 25° produces relatively rapid (half-time about 10 min) desensitization (inactivation) of about 60% of the β-adrenergic receptor binding sites. The desensitized receptors no longer bind the specific β-adrenergic ligand (−)[^{3}H]dihydroalprenolol. The decrease in the number of functional β-adrenergic receptors is also manifest as a decreased ability of isoproterenol to stimulate the membrane-bound adenylate cyclase.**

Desensitization of the β-adrenergic receptors by β-adrenergic agonists in this cell-free system was time and concentration dependent. Moreover, occupancy of the β-adrenergic receptors by ligand was necessary but not sufficient for desensitization. Thus, only agonists and not antagonists produced desensitization. Antagonists also blocked the ability of agonists to desensitize. Partial agonists (which have reduced ability to stimulate adenylate cyclase) caused partial desensitization.

Desensitized receptors were rapidly and completely resensitized by exposure of membranes to guanosine 5′-(β,γ-imino)triphosphate [Gpp(NH)p] and other guanine nucleotides. The specificity of the nucleotide resensitization effect [Gpp(NH)p > GTP > GDP > GMP > ITP > UTP] was essentially identical to that of the "nucleotide regulatory sites" on the adenylate cyclase enzyme in these membranes, which has been previously defined. ATP was also active but was not as effective as Gpp(NH)p or GTP.

These observations suggest that agonist-induced "coupling" of β-adrenergic receptors and adenylate cyclase leads to conformational alterations in the receptors that produce desensitization. The desensitized receptors presumably remain coupled to the enzyme. Alterations in the conformation of adenylate cyclase induced by guanine nucleotides apparently lead to conformational alterations in the receptors that produce resensitization.

Reports from several laboratories have indicated that exposure of a variety of cell types to β-adrenergic catecholamines leads to "desensitization" of the membrane-bound adenylate cyclase to subsequent stimulation by catecholamines (1–8). This desensitization is quite specific, since the ability of other effectors, such as prostaglandins, to activate the adenylate cyclase is unaffected. Desensitization could involve alterations at a number of points in the chain of events leading from β-receptor occupancy to enzyme stimulation. The great specificity of the effects, however, suggested the possibility that alterations in the β-adrenergic receptors might be involved.

Recently, we reported a striking decrease in the number of functional β-adrenergic receptor binding sites in membranes from frog erythrocytes that had been "desensitized" by exposure of intact cells to the β-adrenergic catecholamine isoproterenol *in vivo* or *in vitro* (4, 5). The decrease in the apparent number of receptors was assessed by binding studies with the radioactively labeled β-adrenergic antagonist (−)[^{3}H]dihydroalprenolol (4, 5, 9–13). The decrease in the number of receptors in the isoproterenol treated cells closely paralleled the loss of catecholamine-sensitive adenylate cyclase (about a 50% decrease in each case). There was no change in the apparent affinity of the remaining functional receptors. The receptor population could be regenerated in association with a resensitization of adenylate cyclase to catecholamine stimulation if catecholamines were removed from the incubation mixture. These processes of desensitization and resensitization were relatively slow, requiring several hours for completion (4, 5) and were unaffected by protein synthesis inhibitors (22). Very comparable findings have now been reported in the rat pineal β-adrenergic system (8).

The molecular mechanisms by which isoproterenol and other β-adrenergic catecholamines regulate the function of β-adrenergic receptors remain unknown. Moreover, the complexity of intact cell systems precludes a detailed biochemical analysis of these phenomena. We now report the development of a *subcellular* system, purified frog erythrocyte plasma membranes, in which catecholamine-induced desensitization of β-adrenergic receptors can be studied.

MATERIALS

The sources of all materials used in these studies have been previously documented (4, 5, 9–13). (−)[^{3}H]Dihydroalprenolol has specific activity of 33 Ci/mmol.

METHODS

Membrane Preparation. "Purified" frog erythrocyte membranes were prepared as previously described (11, 12).

Desensitization of β-Adrenergic Receptors in Membranes. The membranes in 75 mM Tris·HCl at pH 8.1 and 10 mM $MgCl_2$ were incubated for 30–60 min at 25°, with different β-adrenergic agents (1–10 μM) with slow shaking. Controls were incubated under identical conditions without added drugs. At the end of incubations the membranes were resuspended in 40 ml of 50 mM Tris·HCl at pH 8.1, 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 use for binding and adenylate cyclase assays.

Resensitization of β-Adrenergic Receptors in Membranes. Control and desensitized membranes were incubated with different nucleotides with and without a "regenerating system"

Abbreviations: Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate; App(NH)p, [adenosine 5′(β,γ-imino)triphosphate; App(CH_2)p, adenosine 5′-(β,γ-methylene)triphosphate; Ap(CH_2)pp, adenosine 5′-α,β-methylene)triphosphate; cAMP, cyclic adenosine 3′:5′-monophosphate; cGMP, cyclic guanosine 3′:5′-monophosphate.

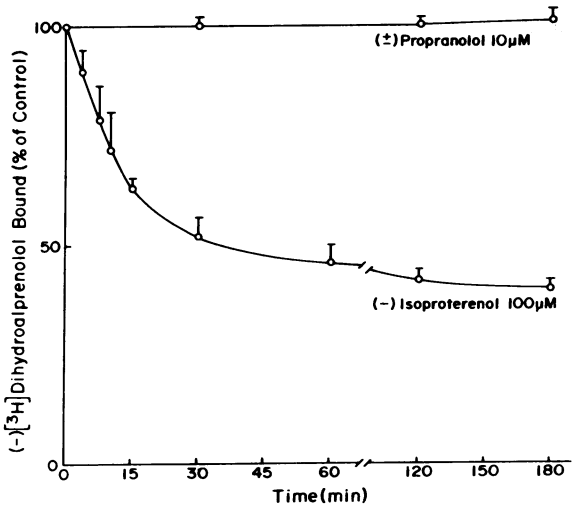

FIG. 1. Time course of reduction in (−)[^{3}H]dihydroalprenolol binding sites in frog erythrocyte membranes preincubated with (−)isoproterenol or (±)propranolol. Membranes were incubated at 25° for the indicated periods and then washed three times with 150 ml of 50 mM Tris·HCl at pH 8.1 and 10 mM $MgCl_2$ prior to performing binding studies. Binding values refer to maximum specific (−)[^{3}H]dihydroalprenolol binding (see *Methods*). The mean (−)[^{3}H]dihydroalprenolol binding to control membranes preincubated without adrenergic agents was 1.48 pmol/mg of protein and did not change significantly over the time periods studied. Values are the means of duplicate determinations ± SEM from six experiments.

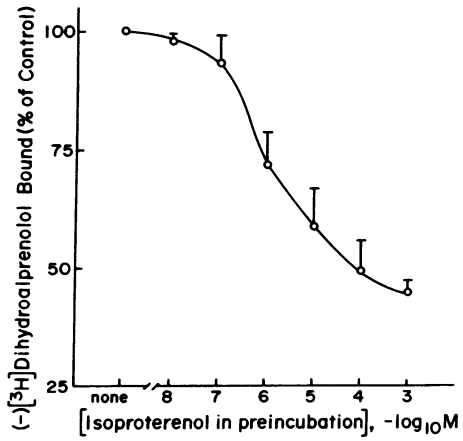

FIG. 2. Concentration dependence of (−)isoproterenol-induced reduction of (−)[^{3}H]dihydroalprenolol binding sites in frog erythrocyte membranes. The membranes were preincubated with the indicated concentrations of (−)isoproterenol for 1 hr at 25°. Controls were preincubated without added drug. Values shown are the maximum specific (−)[^{3}H]dihydroalprenolol binding and represent mean ± SEM of duplicate determinations from three experiments. Note that the ordinate does not extend to zero. Control binding was 1.24 pmol/mg of protein.

(5 mM phosphoenolpyruvate, 40 μg/ml of pyruvate kinase, and 20 μg/ml of myokinase) at 37° for 5 min and then used for binding studies.

Adenylate Cyclase. Assays were performed as described previously (9, 10) and the [^{32}P]cAMP was isolated according to the method of Salomon *et al.* (14).

(−)[^{3}H]Dihydroalprenolol Binding Assay of β-Adrenergic Receptors. Assays were performed as described previously (15), using a rapid glass fiber filter technique to separate receptor bound from free ligand.

In most of these assays high concentrations of (−)[^{3}H]dihydroalprenolol were used (about 2×10^{-7} M) so as 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 the β-adrenergic receptors in these membranes occurs at (−)[^{3}H]dihydroalprenolol concentrations of about 5×10^{-8} M (4, 5, 13). In separate experiments we documented that the high concentrations of radioligand used in these assays fully saturated the receptors even in the presence of isoproterenol concentrations as high as 5×10^{-6} M.

Proteins. Determinations were performed by the method of Lowry *et al.* (16).

RESULTS

Time and Concentration Dependence of Desensitization of β-Adrenergic Receptors in Frog Erythrocyte Membranes. Desensitization of β-adrenergic receptor binding sites by incubation of frog erythrocyte membranes with isoproterenol at room temperature was a fairly rapid process. As shown in Fig. 1, in the presence of 0.1 mM isoproterenol the one-half time for the decrease in (−)[^{3}H]dihydroalprenolol binding was 10–15 min, and the process was essentially complete by 60 min. At lower concentrations of isoproterenol the process occurred more slowly (data not shown). The time course is much more rapid than that previously observed in intact cells, where catecholamine-induced decreases in β-adrenergic receptor binding take several hours for completion (4, 5). The time course is, however, still considerably slower than that for activation of adenylate cyclase by catecholamines, which is complete within seconds (13). It should also be noted that as with intact cells (4, 5) the maximum decrease in apparent receptor number is 50–60% and does not reach 100%.

As shown in Fig. 2, receptor desensitization was dependent on the concentration of isoproterenol. Half-maximal desensitization occurred at 1 μM isoproterenol. This concentration

Table 1. Stimulation of adenylate cyclase by (−)isoproterenol, prostaglandin E_1, and NaF in frog erythrocyte membranes preincubated with (−)isoproterenol or (±)propranolol

Preincubation conditions	n	Adenylate cyclase activity, % above basal		
		Isoproterenol-stimulated	Prostaglandin-stimulated	Fluoride-stimulated
Control	15	302 ± 46	110 ± 33	949 ± 176
(−)Isoproterenol, 100 μM	15	191 ± 29*	108 ± 24	908 ± 166
(±)Propranolol, 10 μM	15	326 ± 22	114 ± 27	960 ± 129

Incubations were for 1 hr at 25°. Controls were incubated without added drugs. Membranes were washed three times as described under *Methods* before the enzyme assays were performed. The basal enzyme activities in control, (−)isoproterenol-, and (+)propranolol-treated membranes were 40 ± 2, 37 ± 1, and 38 ± 1 pmol/min per mg of protein. n = number of experiments. Values shown are mean ± SEM.
* $P < 0.001$ (Students' t test).

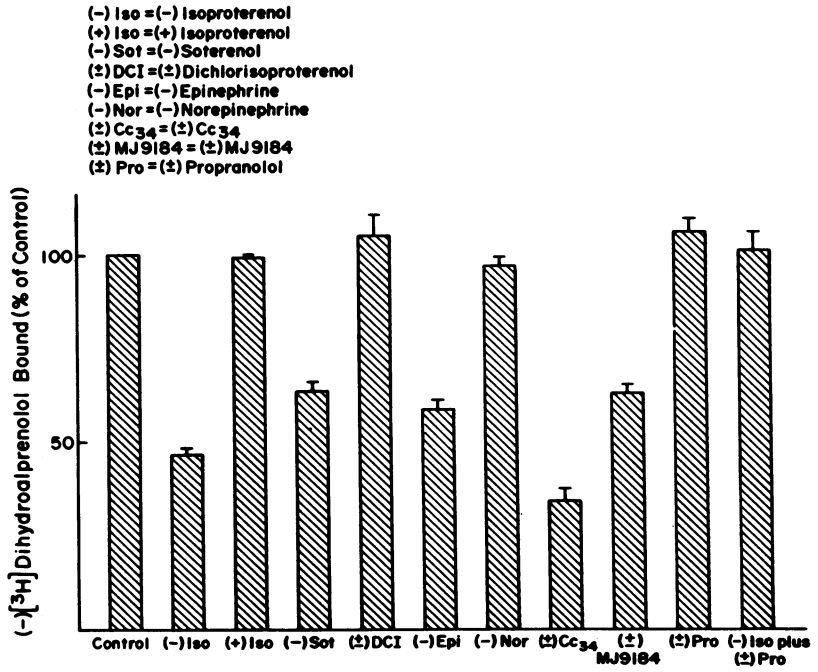

FIG. 3. Effect of preincubation of frog erythrocyte membranes with various β-adrenergic agents on the number of (−)[^{3}H]dihydroalprenolol binding sites. Preincubations were for 1 hr at 25°, following which membranes were washed as described under *Methods*. All agents were present at 10 μM except for (±)Cc_{34} and (±)MJ9184-1, which were present at 1 μM. These agents have previously been demonstrated (11) to occupy essentially all the β-adrenergic receptor binding sites at this concentration. Control (−)[^{3}H]dihydroalprenolol binding was 1.54 pmol/mg of protein. Values shown are the means ± SEM of duplicate determinations from three experiments.

corresponds closely to that which causes half-maximal occupancy of the β-adrenergic receptors and half-maximal stimulation of adenylate cyclase in the membranes (11, 13).

Desensitization of Adenylate Cyclase in Frog Erythrocyte Membranes. Table 1 shows the results obtained when membranes preincubated with isoproterenol and then washed were tested for adenylate cyclase activity. A statistically significant ($P < 0.001$) and selective decrease in catecholamine-sensitive activity was observed. Fluoride- and prostaglandin-sensitive activities were unaltered, as was the basal enzyme activity.

The decrease in catecholamine-stimulated activity, about 37%, though highly statistically significant, was still somewhat less than the decrease in number of β-adrenergic receptors (50–60%). It was also less than the decrease in catecholamine-sensitive adenylate cyclase previously observed when whole cells were exposed to catecholamines *in vivo* or *in vitro* (50–60%) (4, 5). The significance of these observations is discussed further below.

It should be noted that the adenylate cyclase studies were complicated by the marked lability of the enzyme in membrane preparations at room temperature. Thus in the course of a 1 hr preincubation as much as 80% of the enzyme activity was lost in both control and catecholamine-treated membranes. In contrast (−)[^{3}H]dihydroalprenolol binding was unaltered in control membranes incubated at room temperature for up to 5 hr.

Specificity of Desensitization. A variety of β-adrenergic agents was tested for ability to desensitize the β-adrenergic receptor binding sites during a 1 hr incubation with the membranes. The results of such studies are summarized in Fig. 3. Several conclusions are apparent from these experiments. First, desensitization requires occupancy of the β-adrenergic receptors. Thus the (+)isomer of isoproterenol, which has only about 1/500th the affinity of (−)isoproterenol for the receptors, did not desensitize at a concentration of 10 μM. Similarly (−)norepinephrine, which has much less affinity than (−)isoproterenol, had only a very weak desensitizing effect. The data in Fig. 2 also support the contention that desensitization is related to receptor occupancy.

A second conclusion is that the ability of β-adrenergic agents to desensitize the receptors in the membranes is related to their "intrinsic activity" (17), i.e., their maximal ability to stimulate adenylate cyclase. This is in turn a reflection of the extent to which the agents promote "coupling" of the receptors and the enzyme. We have previously determined the intrinsic activities of a wide variety of agents for stimulation of frog erythrocyte membrane adenylate cyclase (11). Agents that have full activity (cause maximal enzyme stimulation equivalent to that observed with isoproterenol) are said to have intrinsic activity = 1. Competitive antagonists have intrinsic activities = 0; partial "agonists" have intermediate activities. Fig. 3 shows that isoproterenol and Cc_{34}, which have full intrinsic activity, cause maximal desensitization. Antagonists such as propranolol and dichlorisoproterenol with intrinsic activities of 0 do not cause desensitization. Moreover, when such antagonists are added to the membranes together with isoproterenol they completely block the desensitizing effects of the agonist. Partial agonists, such as soterenol and MJ9184 (intrinsic activity about 0.25), cause only partial desensitization even at concentrations fully occupying the β-adrenergic receptors in the membranes (11) (Fig. 3).

Recently we have described procedures for solubilizing in an intact form the β-adrenergic receptor binding sites from frog erythrocyte membranes (12) with the detergent digitonin. In these solubilized preparations the β-adrenergic receptors and adenylate cyclase are "uncoupled," and agonists do not produce enzyme stimulation. When such solubilized receptors were exposed to 0.1 mM isoproterenol for up to 2 hr and then dialyzed free of the drug, desensitization was not observed†.

† M. G. Caron, and R. J. Lefkowitz, unpublished observations.

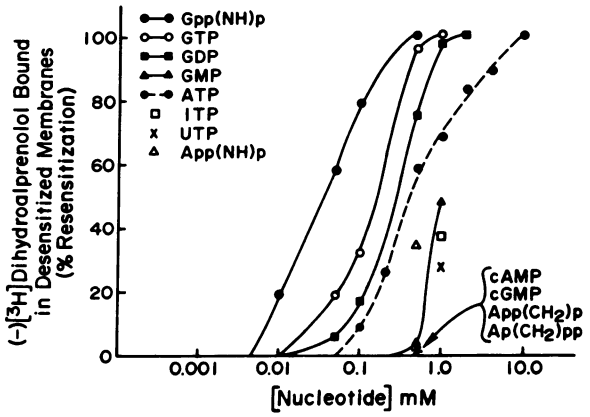

FIG. 4. Resensitization of β-adrenergic receptors [(−)[³H]dihydroalprenolol binding sites] by Gpp(NH)p and other nucleotides in frog erythrocyte membranes. Membranes were desensitized for 1 hr with 0.1 mM (−)isoproterenol, washed three times, then incubated for 5 min at 37° with nucleotides at indicated concentrations before the (−)[³H]dihydroalprenolol binding assays were performed. Control binding was 1.65 pmol/mg of protein and fell to 0.67 pmol/mg of protein in the isoproterenol-desensitized membranes. When desensitized membranes were incubated for 5 min at 37° without nucleotides, no increase in binding sites occurred. Percent resensitization refers to:

(Binding after nucleotide treatment − Binding in desensitized membranes)/(Control binding − Binding in desensitized membranes)

Values shown are means of duplicate determinations from three experiments, except the data for ATP, which are the means of six experiments.

Resensitization of the β-Adrenergic Receptors by Guanosine 5′-(β,γ-imino)triphosphate [Gpp(NH)p] and Other Purine Nucleotides. As noted earlier, the extent of adenylate cyclase desensitization to catecholamines in these studies was less than the decrease in the number of β-adrenergic receptors. Since the only difference between the adenylate cyclase assays and the binding assays is the presence of 1.5 mM ATP, 0.1 mM cAMP, and an ATP-regenerating system in the cyclase assays, we speculated that these reagents might in some way be capable of "resensitizing" the β-adrenergic receptors. That this was in fact the case is documented by the data in Fig. 4. The addition of ATP or other purine nucleotides with or without the regenerating system (pyruvate kinase, myokinase, and phosphoenolpyruvate) to "desensitized" membranes was associated with an increase in the number of β-adrenergic receptor binding sites toward control levels. cAMP at 0.1 mM was without effect, as was the regenerating system alone.

The data in Fig. 4 indicate that in addition to ATP a variety of other nucleotides and nucleotide analogs were capable of resensitizing the β-adrenergic receptors. Gpp(NH)p was most potent in this regard, followed by GTP. The order of potency was Gpp(NH)p > GTP > GDP > ATP > App(NH)p > GMP > ITP > UTP. The nucleotides 3′:5′-cGMP, cAMP, App(CH_2)p, and Ap(CH_2)pp were not active in resensitizing the receptors at the highest concentrations tested, 0.5 mM. Although Gpp(NH)p and other nucleotides increased binding in the desensitized membranes, they were without effect on the binding in control membranes.

In separate experiments membranes were desensitized by exposure to isoproterenol and then washed free of the drug, and incubations were continued in the presence of 10 μM propranolol (without added nucleotide) for several hours. In contrast to the situation previously observed with intact cells (18), such treatment did not lead to regeneration of the β-adrenergic receptors [(−)[³H]dihydroalprenolol binding sites].

DISCUSSION

On the basis of our observations we propose the following model to explain the phenomena of β-adrenergic receptor desensitization and resensitization in this cell-free system. Combination of an agonist with the receptors leads to "coupling" of the agonist–receptor complex with adenylate cyclase. The coupling has two consequences: an immediate conformational alteration of the enzyme, which results in increased adenylate cyclase activity, and a slower conformational alteration in the receptors, which produces "desensitization." In this formulation coupling of receptor and enzyme is required for desensitization to occur. Thus, antagonists that do not lead to coupling do not produce desensitization. Partial agonists that lead to partial coupling and submaximal enzyme stimulation produce only partial desensitization. Receptors in the soluble state, which are functionally uncoupled from the adenylate cyclase, cannot be desensitized.

Guanine nucleotides are capable of occupying regulatory sites on the adenylate cyclase enzyme (19–21). The specificity of the "resensitization" effect is such as to suggest that it may also be mediated by these same nucleotide regulatory sites. Because the desensitized receptors are still coupled to the enzyme, conformational alterations in the enzyme caused by occupancy of the nucleotide regulatory sites also lead to conformational alterations in the receptors. These conformational alterations reactivate the desensitized receptors.

Previous studies of catecholamine desensitization in intact cells had indicated that alterations in the β-adrenergic receptors were involved in the desensitization process (4, 5). Such studies, however, shed little light on the molecular mechanisms involved in these receptor alterations. The demonstration of rapid receptor desensitization and resensitization in a cell-free system sharply limits the possible mechanisms and seems to point strongly toward conformational alterations in the receptors as responsible for the receptor desensitization.

The fact that the reduction in catecholamine-sensitive adenylate cyclase (37%) in the desensitized membranes is less than the reduction in number of β-adrenergic receptors (about 55%) is presumably due to the presence of high concentrations of ATP in the enzyme assays, which partially resensitizes the receptors. In previous studies in which intact frog erythrocytes were desensitized by prolonged (hours) exposure to catecholamines *in vivo* or *in vitro* (4, 5), enzyme desensitization (50–60%) was quite comparable to the reduction in the number of β-adrenergic receptors (50%).

This suggests that with time, at least in intact cells, additional processes occur that are capable of converting the desensitized state of the receptors from a readily reversible one to a more slowly reversible state. The nature of such processes is currently unknown.

As noted, these phenomena cannot be studied in solubilized preparations. Solubilized and purified preparations of the receptors will be valuable tools for learning certain of the molecular characteristics of the receptors. However, for studies of receptor regulation by catecholamines, membrane preparations may well represent the ideal model system.

Since nucleotides are present in intact cells such as erythrocytes at concentrations sufficient to "resensitize" the receptors, desensitization in the intact cells must represent the net result of ongoing desensitization and resensitization processes. By contrast, in the cell-free system employed in these studies nucleotides are absent. This presumably accounts for (*i*) the much more rapid desensitization observed in the membranes as opposed to intact cells, and (*ii*) the resensitization observed in

intact cells but not membranes when isoproterenol is removed from the receptors.

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