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Catecholamine-Induced Subsensitivity of Adenylate Cyclase Associated with Loss of β-Adrenergic Receptor Binding Sites

[(−)[^{3}H]alprenolol/stereospecific binding/isoproterenol/frog erythrocytes]

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ABSTRACT **Injection of frogs with β-adrenergic catecholamines for 1–24 hr produces marked subsensitivity of the erythrocyte membrane adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] to *in vitro* stimulation by isoproterenol. The subsensitization is specific for catecholamine stimulation, since basal and fluoride-stimulated enzyme activity are unaffected. Maximum isoproterenol-stimulated adenylate cyclase activity declines by 75% in the isoproterenol-treated animals ($P < 0.001$). The concentration of isoproterenol causing $^1/_2$ maximal activation of adenylate cyclase, however, is unaltered. (−)[^{3}H]Alprenolol, a potent competitive β-adrenergic antagonist, was used to study directly the β-adrenergic receptor binding sites in the erythrocyte membranes from control and subsensitized animals. A highly significant ($P < 0.005$) 60% fall in the number of the β-adrenergic receptor binding sites ("specific" (−)[^{3}H]alprenolol binding sites) in the treated animals was found. The binding affinity of the sites was not markedly altered. These data suggest that β-adrenergic catecholamines are able to regulate catecholamine sensitivity of tissues *in vivo*, by regulating the properties of the β-adrenergic receptor binding sites.**

The role of receptors and the adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] system in mediating the effects of hormones on target cells is now well established (1). Many hormone and drug receptors have been recognized as distinct specific chemical entities and serious attempts are being made to establish their detailed physicochemical and physiological properties (2, 3). Attempts to identify and isolate β-adrenergic receptors have, until quite recently, been hampered by the lack of a highly specific radiolabeled β-adrenergic ligand which could be used to identify the receptors.

Recently, work from this laboratory has demonstrated the feasibility of labeling β-adrenergic receptors in membrane fractions with (−)[^{3}H]alprenolol, a potent competitive β-adrenergic antagonist (4–8, †). The binding of this radiolabeled ligand to sites in erythrocyte and other membranes has all of the properties (stereospecificity, affinity, kinetics) to be expected of binding to physiological β-adrenergic receptors.

In this communication, these new methods for directly studying β-adrenergic receptors are used to shed new light on the mechanisms of drug-induced "tolerance" or subsensitivity. The model system used is the adenylate cyclase of the frog erythrocyte, which is coupled to a typical β-adrenergic receptor (9–12). It is demonstrated that chronic injection of frogs with β-adrenergic catecholamines leads to a striking and selective decrease in the maximum response of the erythrocyte membrane adenylate cyclase to catecholamines, without alteration of the half maximally effective concentration of drug. This subsensitivity to catecholamines is accompanied by a parallel fall in the number of specific β-adrenergic receptor binding sites in the membranes.

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MATERIALS

(−) and (+)alprenolol hydrochloride were obtained from Hassle. (−) and (+)propranolol hydrochloride were from Ayerst. The (−) isomers of hydrochloride or bitartrate salts of isoproterenol, epinephrine and norepinephrine, as well as cyclic AMP, ATP, phosphoenolpyruvate, and myokinase were from Sigma. The (+) isomers of the bitartrate salts of isoproterenol, epinephrine, and norepinephrine were from Winthrop. Phentolamine was from Ciba and phenoxybenzamine was from Smith, Kline, and French. Pyruvate kinase was from Calbiochem. [^{3}H]cAMP (1–5 Ci/mmol), and [α-^{32}P]-ATP (10–20 Ci/mmol) were from New England Nuclear. (−)Alprenolol was tritiated at New England Nuclear by catalytic reduction with tritium gas using paladium as the catalyst ((−)[^{3}H]alprenolol, specific activity 17 Ci/mmol). "(−)[^{3}H]Alprenolol" has been used throughout this manuscript to identify the compound resulting from catalytic reduction of (−)alprenolol with tritium. (−)Alprenolol contains an unsaturated bond in the aliphatic chain on the 2 position of the aromatic ring. The compound, therefore, might be appropriately referred to as "(−)[^{3}H]dihydroalprenolol." The nature of the labeling process, however, is such that tritium exchange might also take place, yielding (−)[^{3}H]-alprenolol. The labeled material used for these studies has biological activity and chromatographic properties identical to those of native (−)alprenolol.

Alumina, neutral grade, was from Nutritional Biochemicals and Dowex AG 50W X8 was from Bio-Rad. Southern grass frogs (*Rana pipiens*) were obtained from Nasco-Steinhilber.

METHODS

Injection of Animals with Catecholamines. Frogs (150–250 g) were injected subcutaneously with 20–50 mg/kg of isoproterenol, or 15–30 mg/kg of norepinephrine, dissolved in amphibian-buffered saline containing 7% gelatin. Control animals were injected with buffered saline containing gelatin. When the period of treatment was 24 hr, frogs were injected four times at intervals of 6–8 hr; for a 6-hr treatment period, animals were injected once or twice, the second time being 1 hr before sacrifice. A 1-hr treatment consisted of only one injection.

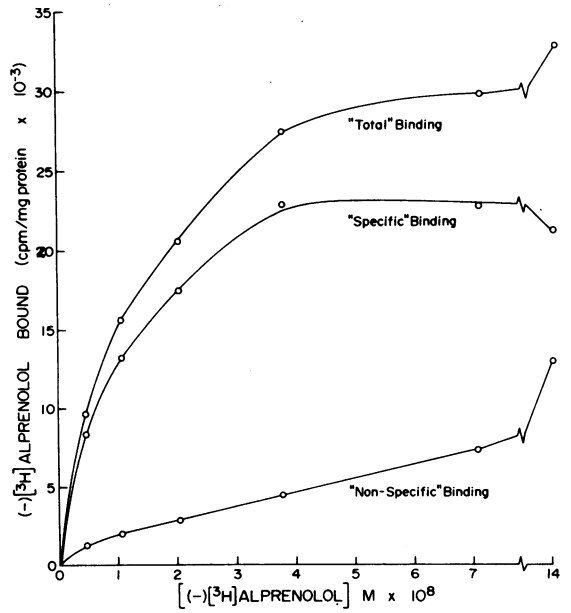

FIG. 1. Dependence of (−)[^{3}H]alprenolol binding to sites in frog erythrocyte membranes on (−)[^{3}H]alprenolol concentration. "Nonspecific" binding refers to that observed in the presence of 10 μM (±)propranolol as described in *Methods*. Each value shown is the mean of duplicate determinations from two experiments.

Membrane Preparations. Heparinized blood from control and treated grass frogs maintained at 23° was collected by cardiac puncture and the red cells were washed four times with a solution of 110 mM NaCl, 10 mM Tris·HCl, pH 7.4. Cells were lysed in water by homogenizing 1 ml of packed cells in 10 ml of H_2O with 10 strokes of a glass-Teflon homogenizer; the lysate was then immediately made 5 mM Tris·HCl, pH 8.1, 2 mM $MgCl_2$, and centrifuged at 30,000 × *g* for 15 min. The pellet was resuspended in 10 mM Tris·HCl, pH 8.1, 10 mM $MgCl_2$ by homogenization and centrifuged at 2,000 × *g* for 10 min over a cushion of the same buffer containing 50% sucrose. The material that sedimented through sucrose was discarded, whereas the supernatant was centrifuged at 30,000 × *g* for 15 min. The pellet was washed twice with the same buffer and finally resuspended in 75 mM Tris·HCl, pH 8.1, 25 mM $MgCl_2$. This fraction was used as the membrane preparation in these studies.

Adenylate Cyclase Assays. Membrane preparations described above were assayed for adenylate cyclase activity. The catecholamine responsiveness of the enzyme in these preparations was examined by performing dose–response curves to isoproterenol over a concentration range from 0.1 μM to 1 mM. Adenylate cyclase assays were performed as described previously (11, 12) and the [^{32}P]cAMP was isolated according to the method of Salomon *et al.* (13).

Binding Assays. Saturation curves of binding of (−)[^{3}H]-alprenolol were performed on membrane preparations from control and treated animals. (−)[^{3}H]Alprenolol binding assays were performed essentially as described earlier (4–8, †). Membrane suspensions (100 μl, 250–500 μg of protein) were incubated with various concentrations of (−)[^{3}H]alprenolol (5–150 nM) in 50 mM Tris·HCl, pH 8.1, 15 mM $MgCl_2$ in a total volume of 150 μl for 10 min at 37°. Duplicate 50 μl samples were pipetted over 300 μl of incubation buffer in microcentrifuge tubes and centrifuged for 1 min in a Beckman Microfuge 152. The surface of the pellets was washed once, and the pellets were dissolved in 0.5 ml of 10% sodium dodecyl sulfate, 10 mM EDTA, and counted in Triton X-100/toluene based scintillation fluid. "Specific binding" was defined as the difference between the amount of radioactivity measured in the presence of (−)[^{3}H]alprenolol alone and that observed in the presence of (−)[^{3}H]alprenolol plus 10 μM unlabeled (±)propranolol (4–8, †). "Nonspecific binding" was usually <20% of the total radioactivity associated with the pellets (Fig. 1). In the results, unless stated otherwise, (−)[^{3}H]alprenolol bound refers to "specific binding" as defined above.

TABLE 1. *Dissociation constants of adrenergic agents for adenylate cyclase coupled β-adrenergic receptors in frog erythrocyte membranes*

Compound	Dissociation constant, μM: (−) [^{3}H]Alprenolol binding	Dissociation constant, μM: Adenylate cyclase
Agonists		
(−)Isoproterenol	2	0.3
(−)Epinephrine	20	15
(−)Norepinephrine	250	150
(+)Isoproterenol	800	700
(+)Epinephrine	600	800
(+)Norepinephrine	1000	—
Antagonists		
(−)Alprenolol	0.015	0.003
(−)Propranolol	0.023	0.003
(+)Alprenolol	0.8	0.17
(+)Propranolol	1.5	0.14
Phentolamine	—	—
Phenoxybenzamine	—	—

Values determined by "(−)[^{3}H]alprenolol binding" represent the concentrations of each agent required to 50% inhibit the binding of (−)[^{3}H]alprenolol (10–20 nM) to the membranes. Values determined in adenylate cyclase assays for agonists are the concentrations necessary for 50% maximal stimulation of the enzyme. For antagonists they represent the concentration necessary to cause a 2-fold rightward shift in the isoproterenol dose–response curve for stimulation of the enzyme (28).—Indicates that an agent was so weak that no meaningful value could be determined. Each value shown is the mean of duplicate determinations from two experiments.

Proteins. Protein determinations were performed by the method of Lowry *et al.* (14).

Statistics. Adenylate cyclase and binding data from control and treated animals were compared by Student's *t* test for paired comparison of means.

RESULTS

Validation of (−)[^{3}H]Alprenolol Binding Assay. The ability of a variety of adrenergic agonists and antagonists to compete for occupancy of the (−)[^{3}H]alprenolol binding sites in the erythrocyte membranes is summarized in Table 1. Also listed in the table are the dissociation constants (K_D) of the agents for the adenylate cyclase coupled β-adrenergic receptors as determined by ability to $^1/_2$ maximally stimulate the enzyme (agonists), or competitively inhibit isoproterenol stimulation (antagonists). It should be noted that certain of these values are slightly higher than those published previously (4). This is presumably a reflection of minor experimental differences.

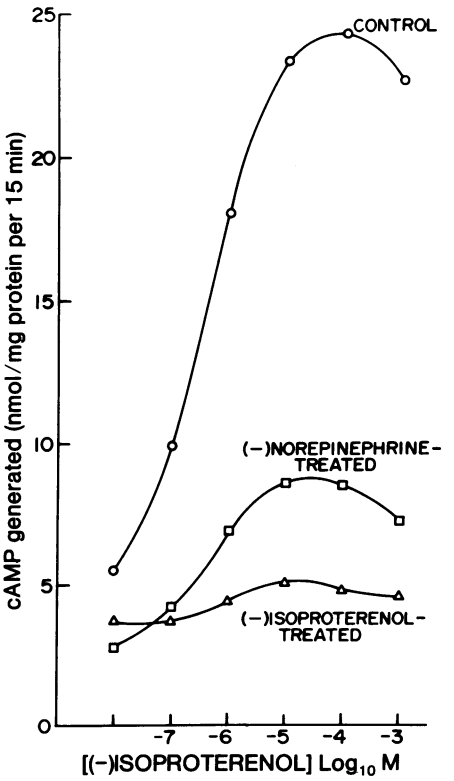

FIG. 2. Isoproterenol stimulation of adenylate cyclase in frog erythrocyte membranes from control, isoproterenol-treated, and norepinephrine-treated animals. The result shown is typical of 10 experiments for isoproterenol and three experiments for norepinephrine. Values are means of duplicate determinations. Drugs were injected over a 24-hr period.

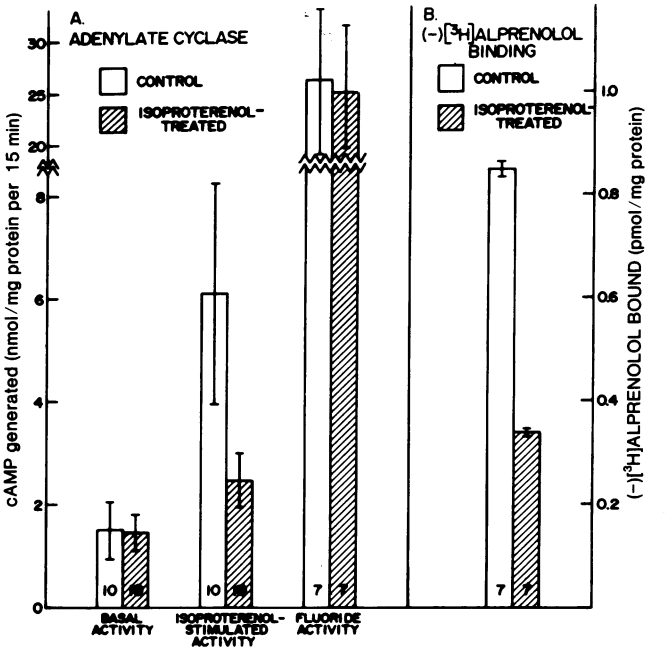

FIG. 3. (A) Basal, isoproterenol-stimulated, and fluoride-stimulated adenylate cyclase in frog erythrocyte membranes from control and isoproterenol-treated animals. Isoproterenol-treated animals received injections over a 24-hr period. Concentrations of activators in the assays were: isoproterenol, 0.1 mM; NaF, 10 mM. Bars represent mean ±SEM of duplicate determinations from 7 to 10 experiments, as indicated. (B) (−)[^{3}H]-Alprenolol binding to frog erythrocyte membranes from control and isoproterenol-treated animals. Isoproterenol was injected over a 24-hr period. In each experiment maximum "specific" binding was determined as indicated in Fig. 1. Bars represent mean ± SEM of duplicate determinations from seven experiments.

In the present studies all incubations were performed at 37° for 10 min, versus 23° for 30 min in ref. 4. In addition, in the present studies "specific binding" was defined as that displaced by 10 μM (±)propranolol, whereas in earlier studies we used 1 μM (±)propranolol. The higher concentration displaces an additional 15–20% of the total (−)[^{3}H]alprenolol binding. It is apparent that affinity for the (−)[^{3}H]alprenolol binding sites directly parallels biological activity as either agonist or antagonist.

Binding of (−)[^{3}H]alprenolol to β-adrenergic receptor binding sites in the membranes was saturable. The data presented in Fig. 1 demonstrate that when increasing concentrations of (−)[^{3}H]alprenolol are added to a fixed amount of membrane protein, "specific" binding reaches a plateau (about 23,000 cpm/mg of protein ≅1.5 pmol/mg of protein). "Nonspecific" binding is only about 15% of total binding and is linearly related to the concentration of (−)[^{3}H]-alprenolol added.

Catecholamine-Induced Subsensitivity of Adenylate Cyclase and β-Adrenergic Receptor Binding. As demonstrated previously, β-adrenergic catecholamines stimulate the frog erythrocyte membrane adenylate cyclase. The dose–response relationship for stimulation of the enzyme by (−)isoproterenol is shown in Fig. 2. Repeated subcutaneous injection of (−)isoproterenol or (−)norepinephrine over a 24-hr period led to a marked decrease in the sensitivity of the enzyme to isoproterenol stimulation (Fig. 2). It should be noted that although the maximum response to isoproterenol is markedly attentuated, the concentration of isoproterenol which caused ½ maximal effects is the same in the treated and control animals. At comparable doses isoproterenol caused greater subsensitivity than norepinephrine.

As demonstrated in Fig. 3A, the decrease in maximum adenylate cyclase stimulation was selective for catecholamine-sensitive activity. Basal and maximum fluoride-stimulated enzyme activity were not significantly different in the treated and control groups. The difference in catecholamine-stimulated activity (% stimulation above basal activity) in the isoproterenol-treated versus control animals was, however, highly significant, $P < 0.001$. Fig. 3B shows the results obtained when (−)[^{3}H]alprenolol binding to membranes from both groups of animals was tested. The results shown represent the maximum specific binding determined by saturation analysis as shown in Fig. 1. A striking fall in the number of (−)[^{3}H]alprenolol binding sites was observed in the membranes from the isoproterenol-treated animals. The difference between the two groups was highly significant, $P < 0.005$. The subsensitization of adenylate cyclase to stimulation by catecholamines could be reproduced in an entirely *in vitro* system by incubating frog erythrocytes at room temperature for 2–10 hr with isoproterenol at concentrations >1 μM. Basal and fluoride-stimulated activity were unaffected.‡

The observed changes in catecholamine-sensitive adenylate cyclase and (−)[^{3}H]alprenolol binding are not due to occupancy of β-adrenergic receptors in the membranes by pre-

‡ J. Mickey and R. J. Lefkowitz, unpublished observations.

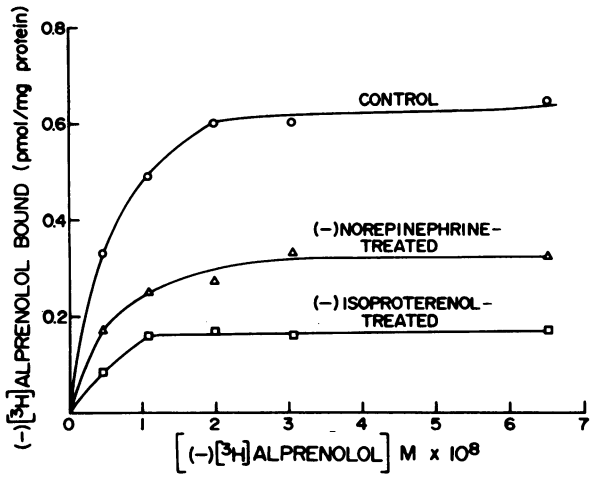

FIG. 4. (−)[^{3}H]Alprenolol binding to frog erythrocyte membranes from control, isoproterenol-treated, and norepinephrine-treated animals. Drugs were injected over a 24-hr period. Values are the means of duplicate determinations from three experiments.

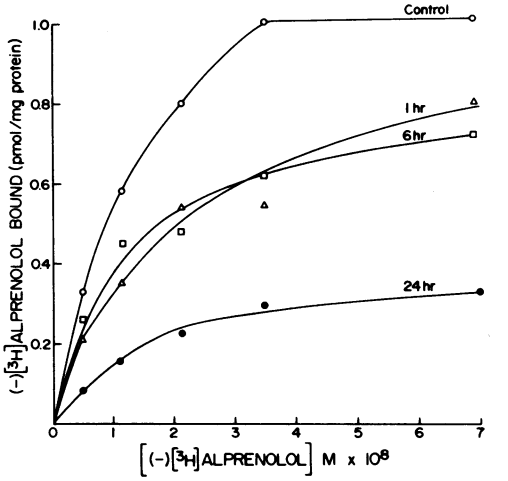

FIG. 5. Time course of reduction in (−)[^{3}H]alprenolol binding sites in frog erythrocyte membranes from isoproterenol-treated animals. Isoproterenol was injected for the indicated time periods (see *Methods*). Values are the means of duplicate determinations from two experiments.

viously injected isoproterenol. First, basal enzyme activity was not elevated in the treated animals as would be expected if significant amounts of isoproterenol remained on the receptors in the membranes. Further, when isoproterenol (0.1 mM) was added to frog blood *in vitro* (allowed to stand for 5 min at 4°) and membranes were prepared in the usual fashion, no subsensitivity was observed. Such control experiments demonstrate that the washing procedures used during preparation of membranes effectively remove isoproterenol from the membranes.

Fig. 4 presents data obtained when the erythrocyte β-adrenergic receptors in control and catecholamine-treated animals were studied directly with (−)[^{3}H]alprenolol. The number of receptors was markedly reduced after 24 hr of (−)isoproterenol or (−)norepinephrine treatment. The decline in the number of receptor binding sites (60% fall, Figs. 3B and 4) was quite consonant with the fall in maximum isoproterenol-stimulated adenylate cyclase (75% fall, Fig. 3A). The affinity of the binding sites appeared unaltered, inasmuch as $^1/_2$ maximal occupancy of the sites occurred at the same concentration (about 10 nM (−)[^{3}H]alprenolol) in treated and control animals.

Maximum subsensitization occurred by 24 hr. More prolonged injection of catecholamines did not lead to more pronounced effects. Shorter periods of treatment (1–6 hr) were associated with lesser degrees of adenylate cyclase subsensitivity to catecholamines and smaller decreases in the number of β-adrenergic receptor binding sites (Fig. 5).

DISCUSSION

"Tolerance," tachyphylaxis, or selective decrease in sensitivity to chronically administered drugs or hormones is now well established, though the molecular mechanisms underlying these phenomena have not been elucidated (15). Recently developed methods for studying hormone and drug receptor binding are now making possible a direct experimental approach to such problems.

Roth and colleagues have demonstrated (16–20) that a variety of insulin-resistant states associated with hyperinsulinemia in man and animals are accompanied by a decrease in the number of specific insulin-binding receptor sites. Decrease in circulating insulin levels is associated with an increase in insulin sensitivity and insulin receptors. The regulatory effects of insulin on the receptors have been reproduced in an *in vitro* system as well (21). Exposure of cultured lymphocytes *in vitro* to insulin for 2–16 hr causes a dose-related decline in the number, but not the affinity of the insulin receptors (assessed by ability to bind ^{125}-labeled insulin).

Chronic exposure to catecholamines is also known to lead to specific subsensitization. An extensively studied system has been the rat pineal gland. Induction of the pineal enzyme serotonin *N*-acetyltransferase by catecholamines appears to be mediated by an adenylate cyclase coupled β-adrenergic receptor (22). Deguchi and Axelrod (23) have demonstrated that injection of rats with isoproterenol leads to a marked subsensitization to the effects of catecholamines. Moreover, the *in vivo* diurnal variation in sensitivity of *N*-acetyltransferase to catecholamines appears to be a reflection of diurnal variations in pineal norepinephrine content, i.e., of the catecholamine levels at the β-adrenergic receptors (24–26).

Similarly, Remold-O'Donnell (27) has demonstrated that a 2-hr exposure of peritoneal macrophages to isoproterenol *in vitro* produces subsensitivity of the adenylate cyclase response to catecholamines. The desensitization is hormone-specific, since the response to prostaglandins is unaffected.

The data reported here demonstrate that injection of frogs with catecholamines produces a marked decrease in the maximum response of the erythrocyte membrane adenylate cyclase to isoproterenol. The frog erythrocyte was selected for these studies because our previous work demonstrated the feasibility of labeling the β-adrenergic receptors in these cells with (−)[^{3}H]alprenolol (4–8, †). Identification and quantification of the β-adrenergic receptors in these cells with (−)[^{3}H]-alprenolol can be made with assurity because of the very low "nonspecific" binding and the identity of the characteristics of the binding sites with those expected of the β-adrenergic receptors (4–8, †).

Our findings indicate that, in the frog erythrocyte system, the induced subsensitivity to isoproterenol constitutes a decrease in maximal stimulated activity rather than an altera-

tion in the K_m for isoproterenol stimulation. The observed effects develop over a period of 24 hr. Subsensitivity was apparent by 1–6 hr and was maximum by 24 hr. The effects seen with isoproterenol were greater than those with norepinephrine. This parallels the greater affinity of isoproterenol for frog erythrocyte β-adrenergic receptors (4). The decrease in apparent number of β-adrenergic receptor binding sites parallels the decrease in adenylate cyclase response.

The apparent subsensitivity of the adenylate cyclase in the treated animals could, at least theoretically, be due to an enhanced rate of catecholamine destruction by membranes from the treated cells. Although this possibility is not absolutely excluded by the data it seems most unlikely. If enhanced catecholamine destruction were occurring, one would not expect the isoproterenol dose–response curves in the treated animals to plateau or even fall at isoproterenol concentrations greater than 10 μM (Fig. 2).

The findings can also not be attributed to residual receptor-bound isoproterenol being carried over into the *in vitro* assays from the injected animals. We demonstrated that addition of isoproterenol to the erythrocytes just prior to preparation and washing of membranes did not mimic subsensitization. Our procedures involve washing the erythrocytes and membrane fractions six times with large volumes of buffer. This would certainly be expected to remove all membrane-bound isoproterenol, since it has previously been demonstrated (29) that the binding of isoproterenol to erythrocyte membranes is rapidly reversible ($t_{1/2}$ < 30 sec). Moreover, persistence of isoproterenol on the receptors would not produce the findings observed in this study. First, basal adenylate cyclase in the treated animals would have been elevated, rather than the same as in controls. Second, (−)[^{3}H]alprenolol binding curves would show a rightward shift in affinity with no apparent change in the number of binding sites. This is because (−)-[^{3}H]alprenolol and isoproterenol compete for the same sites. As the (−)[^{3}H]alprenolol concentration was raised, it would displace the residual isoproterenol so as to finally give the same saturation value. In separate experiments we have demonstrated that this is precisely the result obtained when (−)[^{3}H]alprenolol saturation curves are performed in the presence of added isoproterenol. Finally, if residual catecholamine were responsible for the findings, the 1- and 6-hr treated animals should have shown even more impressive reduction in receptors than the 24-hr treated animals, since they received isoproterenol in closer proximity to the time of sacrifice. As noted, this was not the case.

These findings are quite comparable to previously reported observations on the insulin receptor (16–21). An advantage of the catecholamine responsive system, however, is that a measurable biologic effect (adenylate cyclase stimulation) can be closely correlated with observed changes in receptor binding. As noted, in the present experiments, a parallel decrease in both variables was observed. Miledi *et al.* (30) have previously reported decreased binding of ^{125}I-labeled bungarotoxin to skeletal muscle, previously exposed to acetylcholine. The decrease in receptor binding was associated with a decrease in the responsiveness of the tissue to acetylcholine.

Our studies do not unequivocally answer the question of whether the decreased catecholamine responsiveness of the adenylate cyclase is due to an actual reduction in the number of β-adrenergic receptors or rather to some conformational change in some of the receptors which renders them "inactive." These results do, however, clearly demonstrate that in addition to their ability to stimulate adenylate cyclase via β-adrenergic receptors, catecholamines also possess the ability to regulate the properties of these receptors. The methods utilized here should be generally applicable to the study of β-adrenergic receptor regulation in various tissues.

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