Subsensitivity of Adenylate Cyclase and Decreased β-Adrenergic Receptor Binding after Chronic Exposure to (−)-Isoproterenol in Vitro*

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SUMMARY

In vitro incubation of frog erythrocytes with (−)-isoproterenol, 0.1 mM, at 23° for 10 to 24 hours caused a 63% decline (p < 0.001) in the maximum (−)-isoproterenol-stimulated adenylate cyclase activity in the erythrocyte membranes. Affinity for (−)-isoproterenol as judged by the concentration which half-maximally stimulated the enzyme was not markedly altered. Basal enzyme activity and stimulation by fluoride or prostaglandin E1 remained unaltered. The number of β-adrenergic receptor binding sites, assessed by binding studies with the β-adrenergic antagonist (−)-[3H]alprenolol, declined by 50% (p < 0.005) in the (−)-isoproterenol-treated cells. The binding affinity of the sites was not changed. Regulation of the concentration of functionally active β-adrenergic receptors in membranes may be one of the mechanisms by which chronic exposure to catecholamines desensitizes tissues to β-adrenergic stimulation.

The phenomenon of tolerance, desensitization, or subsensitivity to certain drugs and hormones has long been observed in both clinical and laboratory settings (1). The molecular mechanisms of these effects, however, are not yet known. We have undertaken the study of catecholamine tolerance using the frog erythrocyte as a source of catecholamine-sensitive adenylate cyclase. Methods for quantitating the hormonal responsiveness of adenylate cyclase and for studying hormone interactions with the β-adrenergic receptor have now been developed in this system (2-7). Binding of the radiolabeled β-adrenergic antagonist (−)[3H]alprenolol to membrane fractions from canine heart (8) and frog erythrocytes demonstrates all of the characteristics expected of binding to the β-adrenergic receptor. The binding is rapid and rapidly reversible and displays ligand specificity, stereospecificity, and affinity appropriate to the β-adrenergic receptors.

Recent investigations (4, 9-12) have shown that exposure of various tissues to catecholamines both in vitro and in vivo results in decreased sensitivity of β-adrenergic-mediated responses to subsequent catecholamine stimulation. This desensitization may occur through any number of changes in the pathway from hormone receptor interaction to generation of cyclic AMP. We now report that chronic exposure of intact frog erythrocytes to (−)-isoproterenol in vitro is associated with a decreased responsiveness of the enzyme to β-adrenergic stimulation; and that furthermore, this loss of sensitivity is accompanied by a decrease in the apparent number of β-adrenergic receptors. Our data suggest that a major mechanism involved in the phenomenon of desensitization may be a decrease in the number of functional β-adrenergic receptors.

Grass frogs were bled by cardiac puncture and the heparinized blood was washed twice in buffered “amphibian saline” (109 mM NaCl/9 mM Tris, pH 7.4). Pooled blood was incubated at 23° with or without 0.1 mM (−)-isoproterenol HCl 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 for 10 to 24 hours. Antibiotics were added to retard bacterial growth which led to cell lysis. Medium was renewed after 12 hours in 24-hour incubation experiments. Cells were shielded from light and kept in suspension by slow rotation.

After 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/2 mM dithiothreitol, pH 7.4) and lysed by quick freezing in an ethanol-Dry Ice bath. Forty milliliters of washing buffer were added and the lysate thawed at 9°. After centrifugation (30,000 × g for 15 min), the pellet was resuspended by homogenization in “cyclase buffer” (75 mM Tris/25 mM MgCl2, pH 7.4) and recentrifuged at 2,000 × g for 8 min over a cushion of cyclase buffer containing 50% sucrose. The supernatant fraction was collected and pelleted at 50,000 × 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 determinations were performed by the method of Lowry et al. (13).

As shown in Fig. 1, preincubation of intact erythrocytes with 0.1 mM (−)-isoproterenol led to a striking (63%) decrease in the subsequent ability of (−)-isoproterenol to stimulate adenylate cyclase in membrane fractions (p < 0.001, paired t test). Affinity of the β receptors for this ligand (as reflected by the concentration of (−)-isoproterenol which caused half-maximal response) was not markedly changed, however. Basal enzyme activity and sodium fluoride stimulation were unaltered, indicating that (−)-isoproterenol preincubation did not have a toxic effect on the adenylate cyclase enzyme itself. Stimulation of the enzyme by prostaglandin E1 also was unaffected by preincubation, showing that of the two hormone receptors coupled to adenylate cyclase that were available for study, the β-adrenergic system was specifically desensitized. Desensitization appeared to be maximum by 10 hours. Twelve- and twenty-four-hour incubations (one and three of the ten experiments) resulted in no further desensitization.

That the observed effect was not an artifact due to persistence of (−)-isoproterenol on the receptors from the preincubation period can be ruled out by the following observations. First, basal activity in treated membranes was not higher than the controls as would be expected if residual drug remained on the
receptors. Second, propranolol (10 μM) did not significantly lower basal activity in treated cells as would be expected if (-)-isoproterenol had not been washed off. Finally, when cells were exposed to the same concentration of (-)-isoproterenol only briefly (<5 min) prior to washing, no desensitization resulted.

Although a number of mechanisms could account for the adenylate cyclase "tolerance" observed, the specificity for (-)-isoproterenol sensitive enzyme activity prompted us to examine the status of the β-adrenergic receptors through binding studies with (-)-[3H]alprenolol. Fig. 2A shows a typical binding curve for saturation of receptor sites with (-)-[3H]alprenolol in control and (-)-isoproterenol-treated cells. In seven experiments maximum binding in desensitized cell membranes was reduced to 50% of control values (p < 0.005, paired t test) (Fig. 2B). Receptor affinity (as reflected by the concentration of (-)-[3H]alprenolol resulting in half-maximal binding) was not significantly changed. That these results were not due to residual (-)-isoproterenol on the receptors is excluded by the arguments noted above plus the following observations. Persistence of (-)-isoproterenol or a metabolite on the receptors would appear to alter the affinity of (-)-[3H]alprenolol binding (shift the saturation curve to the right) without lowering the apparent number of binding sites (maximum binding). In separate experiments, we have demonstrated that this was precisely the result obtained when (-)-isoproterenol, 0.5 to 5 μM, was actually added to control membranes prior to performing (-)-[3H]alprenolol binding studies.

These experiments demonstrate that tolerance to β-adrenergic stimulation induced in vitro is characterized by both a specific decrease in maximum (-)-isoproterenol-stimulated adenylate cyclase activity and a loss of β-adrenergic receptor binding capacity, with no evidence for change in affinity. Other investigators in our laboratory have found that repeated injection of frogs with β-adrenergic agonists over a 24-hour period results in changes in adenylate cyclase sensitivity and in number of receptors that are similar to those we have observed in vitro (4). Although the 50% reduction in receptor binding sites closely parallels the 63% reduction in catecholamine-sensitive cyclase activity, drug tolerance in this model system may be the result of factors other than decreased hormone-receptor interaction, such as alterations in receptor-enzyme coupling. Among the possible explanations for the reduction in receptor binding observed are a true drop in the number of receptors (decreased receptor synthesis, increased receptor degradation) or an inactivation of a portion of the receptor binding sites (conformational change, irreversible binding of a metabolite, or negative cooperativity among receptors). We have only ruled out the possibility that residual (-)-isoproterenol or some reversibly binding degradation product remained from the original preincubation period.

Footnote: 1 The abbreviation used is: cAMP, cyclic adenosine 3’:5’-monophosphate.
The present data do not distinguish among the other possibilities noted above.

In the cholinergic system, investigators have shown that prolonged exposure of muscle tissues to acetylcholine resulted in decreased muscular responsiveness to acetylcholine (changes in miniature end-plate potential) and in a decreased number of cholinergic receptors (assessed by α-bungarotoxin binding studies) (15). The characteristics of desensitization in this electrophysiologic system closely parallel our findings in an adrenergic receptor-linked enzyme system.

In the field of catecholamine research, investigators have shown that preincubation of thymocytes (9) and macrophages (10) and fibroblasts (12) with catecholamines induced a decreased response of adenylate cyclase to a subsequent β-adrenergic stimulation. However, these studies did not distinguish between a change in hormone affinity or in maximum enzyme velocity. In addition, neither direct receptor binding studies nor other investigations of mechanism were reported. Thus, our studies go beyond these in vitro investigations by correlating catecholamine-induced desensitization of adenylate cyclase with a reduction in the number of functional adrenergic receptors, suggesting a possible mechanism for the induction of catecholamine tolerance. Further work using the system reported here is required to characterize fully the induction of this phenomenon and explore the molecular mechanisms through which it evolves.

REFERENCES

Subsensitivity of adenylate cyclase and decreased beta-adrenergic receptor binding after chronic exposure to (minus)-isoproterenol in vitro.
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