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SHORT COMMUNICATION

A Desensitized State of the *Beta* Adrenergic Receptor Not Associated with High-Affinity Agonist Occupancy

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SUMMARY

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Exposure of certain cells (e.g., frog erythrocytes) to *beta* adrenergic agonists leads to desensitization of the membrane-bound adenylate cyclase to further *beta* adrenergic stimulation, which is associated with a fall in the number of *beta* adrenergic receptor binding sites. To explain further the mechanism of this agonist-induced desensitization of adenylate cyclase-coupled *beta* adrenergic receptors, intact frog erythrocytes were "desensitized" by incubation with the radiolabeled *beta* adrenergic agonist [^{3}H]hydroxybenzylisoproterenol. This incubation with agonist led to a "loss" of 33% of the (−)-[^{3}H]dihydroalprenolol binding sites (*beta* adrenergic receptors) from membrane fractions prepared from the erythrocytes. Although 192 fmoles/mg of protein of (−)-[^{3}H]dihydroalprenolol binding sites were lost from the membranes of desensitized cells, only 24 fmoles/mg of protein of [^{3}H]hydroxybenzylisoproterenol remained specifically bound to sites in the membranes from the desensitized cells. Thus residual agonist, tightly bound to "high-affinity" receptor sites, does not explain the loss of *beta* adrenergic receptor binding sites that occurs during desensitization of the intact cells.

Hormonal control of adenylate cyclase systems has been the subject of a great deal of investigative work over the past 15 years (1). Until recently the focus of much of this work was on the delineation of the mechanisms of activation of the enzyme adenylate cyclase by agonist hormones and drugs. More recently another aspect of hormonal regulation of adenylate cyclase activity has been increasingly studied: the desensitization of the adenylate cyclase system by agonists (2). *Beta* adrenergic receptor-coupled adenylate cyclase systems have been widely used for studies of both activation and desensitization of adenylate cyclase. In a number of systems catecholamine stimulation of adenylate cyclase becomes attenuated with time during continued exposure to catecholamine agonists (3-11). Desensitization is often receptor-specific (4, 6, 8, 9, 11). This is the case in the frog erythrocyte system, where exposure of intact frog erythrocytes to an agonist, such as isoproterenol, leads to desensitization of the catecholamine-sensitive adenylate cyclase without effects on basal or fluoride- or prostaglandin E_1-sen-

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sitive enzyme activity (12, 13).

In the frog erythrocyte model system the desensitization to *beta* adrenergic stimulation induced by exposure of the cells to isoproterenol is associated with a 30–60% decline in the maximum number of *beta* adrenergic receptors in the cell membranes that can be assayed by (−)-[^{3}H]dihydroalprenolol binding (8, 12–14). Several data have suggested that the agonist-induced changes in receptor binding were likely mediated by agonist-induced inactivation of the receptors, presumably by conformational changes (13, 14). For example, the desensitization and decrease in binding sites are reversible, and this reversibility is not impaired by inhibitors of protein synthesis (14).

Exposure of isolated membranes to agonist catecholamines also leads to a decrease in *beta* adrenergic receptor number as assayed by (−)-[^{3}H]dihydroalprenolol binding (15, 16). Quite recently we have demonstrated that this agonist-induced fall in receptor number in membranes is due to the agonist-induced formation of a high-affinity state of the receptor, which tightly binds the agonist (17, 18). The agonist therefore appears to act as a noncompetitive, irreversible antagonist, as shown by subsequent dihydroalprenolol binding to the receptors. This was demonstrated by using both a radiolabeled agonist ([^{3}H]hydroxybenzylisoproterenol) and the radiolabeled antagonist [^{3}H]dihydroalprenolol to perform the binding studies (18).

There are striking differences (time course, reversibility by nucleotides) in the properties of the agonist-induced "fall" in receptor number which we observed in whole cells and that which we observed in membranes. In view of these differences, we wondered whether formation of a high-affinity state of the receptor, with persistent tight occupancy of the receptors by bound agonist, was sufficient to explain the loss of receptor binding capacity induced in whole cells by prior exposure to agonist, or whether additional mechanisms would need to be invoked. We report here the results of experiments in which intact frog erythrocytes were desensitized by exposure to a radiolabeled agonist, [^{3}H]hydroxybenzylisoproterenol. The results demonstrate that when whole cells are desensitized in this fashion (followed by extensive washing and preparation of washed membranes) tight binding of agonist to receptors does not explain the decrease in *beta* receptor number.

The radioligands used in this study were [^{3}H]hydroxybenzylisoproterenol, an agonist with specific radioactivity of 20 Ci/mmole, and (−)-[^{3}H]dihydroalprenolol, an antagonist with specific radioactivity of 33 Ci/mmole. Both ligands were obtained from New England Nuclear. We have previously described the purity and biological activity of these agents (17, 19). The sources of all other materials used have been described previously (13, 14).

Intact frog erythrocytes obtained from Southern jumbo grass frogs (Nasco-Steinhilber) were desensitized by incubation with agonist as previously described (13). Instead of isoproterenol, however, incubations were performed for 2.5 hr at 25° with 0.1 μM [^{3}H]hydroxybenzylisoproterenol in a buffer of 101 mM NaCl, 10 mM dextrose, 17 mM Tris-HCl, and 0.2 mM sodium metabisulfite, pH 7.4, containing 1 mM catechol and 0.1 mM ascorbic acid. All incubations were shielded from light. At the completion of these preliminary incubations the whole cells were washed three times by resuspension in 40 ml of buffer consisting of 100 mM NaCl and 10 mM Tris-HCl, pH 7.4, containing 1 mM catechol and 0.1 mM ascorbic acid, and centrifuged at 1800 × g for 5 min.

Purified frog erythrocyte plasma membranes were prepared as previously described from the washed frog erythrocytes, except that cells were lysed by freezing in liquid nitrogen and then thawing. The purified membrane fraction was washed an additional two or three times by resuspension in 40 ml of buffer containing 129 mM NaCl, 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, 2 mM dithiothreitol, 1 mM catechol, and 0.1 mM ascorbic acid, followed by centrifugation at 30,000 × g prior to performing binding assays.

[^{3}H]Dihydroalprenolol binding assays were performed by incubating aliquots of

membranes (approximately 70 μg of protein) in a volume of 150 μl with saturating concentrations of [^{3}H]dihydroalprenolol (approximately 100 nM) for 10 min at 37°. In all experiments replicate samples of membranes were also incubated with the radioligand in the presence of 10 μM (±)-propranolol to determine nonspecific binding. Specific or *beta* receptor binding was then determined as the difference between total binding and nonspecific binding. Other samples of membranes from desensitized cells were filtered after incubation without any added radioligand, and the residual radioactivity remaining from the preliminary incubations was determined. The Whatman GF/C filter discs used for the filtration step of the assay were placed in scintillation counting vials and allowed to dry overnight. Then 10 ml of a Triton X-100–toluene-based fluor were added, and the samples were counted in a Packard liquid scintillation spectrometer at an efficiency of 40%.

Proteins were determined by the method of Lowry *et al.* (20), and statistical comparison of group means was evaluated by Student's *t*-test for unpaired data. $p < 0.05$ was taken as a statistically significant difference.

In these experiments three groups of cells were incubated. The first group was incubated without added drug. The second, "desensitized" group was incubated with 0.1 μM [^{3}H]hydroxybenzylisoproterenol. The third group of cells was incubated with 0.1 μM [^{3}H]hydroxybenzylisoproterenol plus 10 μM (±)-propranolol. We have previously demonstrated that this concentration of propranolol is sufficient to block the desensitization caused by the agonist (12).

It was found that binding of [^{3}H]dihydroalprenolol to membranes from groups 1 and 3 was not significantly different, as was anticipated. However, [^{3}H]dihydroalprenolol binding to membranes from cells of group 2, which had previously been incubated with the radiolabeled agonist, was significantly reduced. In 12 experiments the concentration of [^{3}H]hydroxybenzylisoproterenol used during preliminary incubations (0.1 μM) reduced subsequent [^{3}H]dihydroalprenolol binding by 33% ± 2% ($p < 0.001$). In the 12 experiments there was a mean loss of 192 ± 3 fmoles/mg of protein of [^{3}H]dihydroalprenolol binding sites. However, when aliquots of membranes from the desensitized cells were counted directly to determine the amount of residual specifically bound [^{3}H]hydroxybenzylisoproterenol, only 24.4 ± 9 fmoles/mg of protein of [^{3}H]hydroxybenzylisoproterenol were found to be tightly bound to the receptors in the membranes (Fig. 1). In these experiments specific residual [^{3}H]hydroxybenzylisoproterenol bound to the receptors was determined as the difference in radioactivity bound to membranes from group 2 vs. those from group 3, in which [^{3}H]hydroxybenzylisoproterenol had previously been incubated with cells in the presence of a high concentration of propranolol. Presumably any [^{3}H]hydroxybenzylisoproterenol bound to membranes after preliminary incubation in the presence of a high concentration of propranolol must be bound to nonspecific, non-receptor-binding sites. It can be seen from Fig. 1 that the difference between the number of [^{3}H]dihydroalprenolol binding sites lost during desensitization and the number tightly bound by the radiolabeled agonist [^{3}H]hydroxybenzylisoproterenol is highly significant at the $p < 0.001$ level.

The present studies do not answer the question of the fate of the *beta* adrenergic receptors in the desensitized cells that can no longer be detected by [^{3}H]dihydroalprenolol binding. That these sites are not actually lost from the membranes has been suggested by the observations that in whole cells the sites can be observed to return over a period of hours when agonist is removed from the system (13, 14), and that this return of receptors does not appear to require new protein synthesis (14). One speculation, which is consistent with the proposed formulation of desensitization of Katz and Thesleff for the nicotinic receptor (21), is that the desensitized receptors represent a conformationally altered state of the receptor which is in some way derived from a high-affinity state of the receptor induced by agonists

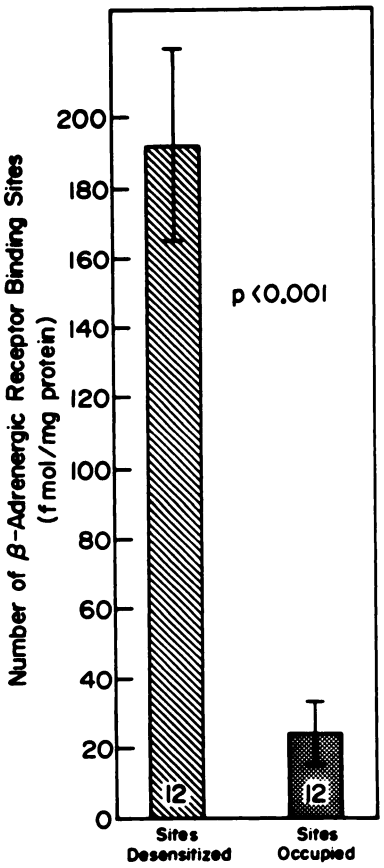

FIG. 1. *Desensitization of beta adrenergic receptors by [³H]hydroxybenzylisoproterenol in frog erythrocytes*

Frog erythrocytes were incubated with 0.1 μM [³H]hydroxybenzylisoproterenol for 2.5 hr in the presence and absence of 10 μM (±)-propranolol as described in the text. Cells were then washed three times and membranes prepared. The membranes were washed an additional three times. "Sites desensitized" refers to the difference in maximal [³H]dihydroalprenolol binding to membranes from control cells and cells previously incubated with [³H]hydroxybenzylisoproterenol. ([³H]Dihydroalprenolol binding to cells previously incubated with [³H]hydroxybenzylisoproterenol and 10 μM propranolol was the same as control.) "Sites occupied" refers to the number of sites in membranes specifically occupied by residually bound [³H]hydroxybenzylisoproterenol from the whole cell preliminary incubations. This was determined as the difference in radioactivity associated with membranes from cells previously incubated with [³H]hydroxybenzylisoproterenol vs. those previously incubated with [³H]hydroxybenzylisoproterenol and propranolol. Values shown represent means ± standard errors of 12 experiments, determined in duplicate.

(22). Previously we have speculated that such a high-affinity state of the receptor is the crucial intermediate, whose stimulation by guanine nucleotides is responsible for the expression of hormone-stimulated adenylate cyclase (18). Inasmuch as the high-affinity state of the receptor is uniquely formed only by agonists, and since desensitization is agonist-specific, it seems reasonable that this state of the receptor might represent some form of precursor of the "desensitized" state of the receptor.

Our studies further underscore the differences between desensitization of intact frog erythrocytes and the process we have termed "*beta* receptor desensitization" of frog erythrocyte membranes (15, 16). The present studies make it seem increasingly unlikely that the process of "receptor desensitization" induced by *beta* agonists when incubated with isolated membranes bears any simple relationship to the desensitization process induced in whole cells. Rather, our current thinking is that the high-affinity state of the *beta* receptor induced by agonists in membranes is more directly involved in the process of activation of adenylate cyclase (18).

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