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# ALTERED STATES OF CARDIAC BETA-ADRENORECEPTORS

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**Abstract**—Beta-adrenergic agonists are believed to alter beta-adrenoreceptors to an active conformation, and sometimes to a desensitized state. Beta-antagonists are thought to bind without causing either activation or desensitization. To examine these states further, the stability of occupied and unoccupied receptors to inactivation by heat and enzymatic digestion was studied. Binding of (−)-[$^3$H]dihydroalprenolol was used as the criterion for receptor stability. Both (−)-isoproterenol (50 $\mu$M) and (±)-alprenolol (20 $\mu$M) markedly stabilized beta-receptors in cardiac membranes to heat. Remarkably, no inactivation was seen in the presence of the agonist during incubations of membranes at 50° for 1 hr, and the binding properties of the receptors were unchanged after removal of isoproterenol. In contrast, both ligands potentiated degradation of beta-receptors by $\alpha$-chymotrypsin. The results provide evidence for three different physical states of cardiac beta-receptors: unoccupied, agonist-occupied, and antagonist-occupied. (No attempt was made to distinguish active from desensitized receptors.) We hypothesize that both ligands may increase protrusion of the receptor protein from the membrane surface, and we suggest that (−)-isoproterenol is a useful agent for stabilizing beta-receptors.

Both agonists and antagonists bind to beta-adrenoreceptors, but only the former initiate the activation of adenylate cyclase [1, 2]. It is therefore believed that only agonists can induce or bind to a form of receptors which is required for activation. Furthermore, agonist binding to beta-receptors can be modified by desensitization [3, 4], thiol reagents [5], magnesium ions [6] or solubilization [7, 8], whereas little or no change in antagonist binding occurs. Consideration of these results has led to suggestions that the beta-receptor protein can exist in at least three types of conformations: resting or basal (unoccupied), active (agonist-occupied), and modified (occupation not necessarily required). The question of whether the simple binding of a ligand, particularly an antagonist, produces another altered state has remained moot.

To examine further the different states of beta-adrenoreceptors, we have studied the stability of occupied and unoccupied receptors to inactivation by heat and enzymatic digestion. Our premise during these experiments was that significant changes in the conformation of the receptor protein would be reflected by increased or decreased stability. The degree of unaltered binding of (−)-[$^3$H]dihydroalprenolol, an antagonist, was used as the criterion for receptor stability. These studies are analogous to previous work concerning the effects of substrates on the stability of enzyme proteins.

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## MATERIALS AND METHODS

*Membrane preparation.* Mongrel dogs of either sex were anesthetized with 30 mg/kg of intravenous pentobarbital. Hearts were removed and rinsed with 0.15 M NaCl, and ventricular tissue was homogenized in 20 vol. of 10 mM Tris/HCl buffer at pH 8.0, containing 5 mM EDTA, for 45 sec in a Waring blender at top speed. Each homogenate was filtered through four layers of cheesecloth, and enough sodium perchlorate was added in 100 ml of homogenization buffer to give a final concentration of 0.15 M. The suspension was re-homogenized at top speed for 15 sec and centrifuged at 480 *g* for 10 min. (All *g* forces are at the bottom of the tubes.) The pellet was discarded and the supernatant fluid was centrifuged at 27,500 *g* for 20 min. The top layer of the resultant bilayered pellet was swirled free with 10 vol. of 50 mM Tris/HCl buffer at pH 8.0, resuspended using a Polytron blender (P10 head, half-maximum speed) for 20 sec, and resedimented. The pellet was resuspended in 10 vol. of 50 mM sodium phosphate buffer at pH 7.5 ("buffer"), re-sedimented, and finally dispersed in 1 vol. of buffer for use. All operations were carried out at 0–4°. Protein was determined by the method of Lowry *et al.* [9], using bovine serum albumin as the standard.

*Beta-adrenoreceptor determinations.* Binding of (−)-[$^3$H]dihydroalprenolol to adrenoreceptors was determined by incubating membranes (0.1 to 0.3 mg protein) in 150 $\mu$l buffer with 0.3 to 20 nM [$^3$H]dihydroalprenolol for 15 min at 25°. At the end of each incubation, 4 ml of ice-cold buffer was added to each tube and the suspension was immediately filtered on a Whatman GF/C glass fiber filter under reduced pressure. Filters were washed with 12 ml of buffer, dried for 30 min at 50°, placed in scintillation

vials with 6 ml of a toluene/Triton X-100 scintillation mixture, and counted for 5 min in a scintillation counter. When isoproterenol was present during incubations, each suspension was also made 0.1% in ascorbic acid to prevent oxidation of the catecholamine. Non-specific binding of [$^3$H]dihydroalprenolol to membrane sites other than beta-adrenoreceptors was assessed in parallel incubations with 3 $\mu$M (±)-alprenolol, and these counts were subtracted from the total bound to give "specific" binding. All assays were done in triplicate; results varied by less than 7 percent.

*Proteolytic digestion.* Cardiac membranes (10 mg of membrane protein) were preincubated in 10 ml of buffer for 15 min at 25° with and without either 20 $\mu$M (±)-alprenolol, or 50 $\mu$M (−)-isoproterenol plus 0.1% ascorbic acid. Enough chymotrypsin was then added to each tube in 100 $\mu$l of buffer to give 100 $\mu$g enzyme/mg of membrane protein. After continued incubation at 25° as indicated in the text, 20 ml of ice-cold buffer was added and the tubes were immediately centrifuged at 48,000 *g* for 10 min. Pellets were resuspended in 30 ml of buffer and recentrifuged. After two more washings by resuspension and sedimentation, the membranes were resuspended in 1 ml of buffer for binding assays.

*Heat treatment.* Membranes (30 mg of membrane protein) were preincubated in 10 ml of buffer for 15 min at 25° with and without either 20 $\mu$M (±)-alprenolol, or 50 $\mu$M (−)-isoproterenol plus 0.1% ascorbic acid. At the end of each incubation, the tubes were transferred to another bath at 50° for the times indicated in the text. Twenty ml of ice-cold buffer was then added and, after centrifugation at 48,000 *g* for 10 min, membranes were washed three times as described for proteolytic digestion. The final pellet was resuspended in 2 ml of buffer for binding assays.

*Materials.* (−)-[$^3$H]Dihydroalprenolol (DHA, 48.6 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA, and $\alpha$-chymotrypsin and (−)- and (+)-isoproterenol from the Sigma Chemical Co., St. Louis, MO. (±)-Alprenolol hydrochloride was a gift from A. B. Hässle Pharmaceuticals, Goteborg, Sweden.

## RESULTS

*Proteolysis of beta-receptors.* Incubation of cardiac membranes with and without 50 $\mu$M (−)-isoproterenol at 25° for 60 min (followed by washing to remove isoproterenol) resulted in equal numbers of beta-receptors, measured with [$^3$H]dihydroalprenolol (results not shown). Figure 1 shows the effects of $\alpha$-chymotrypsin on the rate of beta-receptor inactivation in the presence and absence of an agonist and antagonist. Under conditions in which unoccupied receptors were relatively resistant to degradation (26 percent lost in 30 min), both the antagonist, alprenolol (58 percent lost), and the agonist, (−)-isoproterenol (69 percent lost), significantly increased the rate of inactivation. In all three circumstances receptor loss appeared to be exponential with time, in keeping with our previous evidence for one population of sites binding [$^3$H]dihydroalprenolol in these membranes [10, 11]. We could find no effects of isoproterenol or alprenolol on the activity of $\alpha$-chymotrypsin in degrading total membrane protein, using the sensitive assay method of Walsh and Wilcox [12] (results not shown).

*Heat stability of beta-receptors.* Figure 2 shows the heat stability of unoccupied, antagonist-occupied and agonist-occupied states of the beta-receptor. During a 60-min incubation period at 50°, 88 percent of the receptor was lost in the unoccupied state and

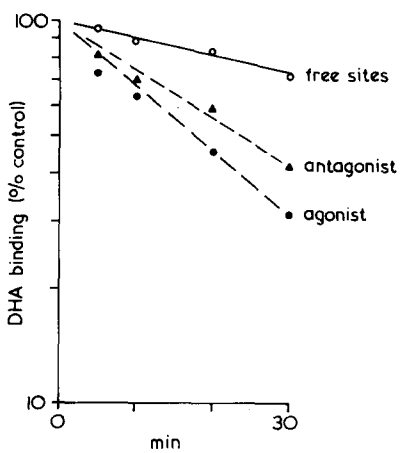


Fig. 1. Effect of $\alpha$-chymotrypsin on the rate of beta-receptor inactivation. Cardiac membranes (1 mg protein/ml) were preincubated with 20 $\mu$M (±)-alprenolol (▲), 50 $\mu$M (−)-isoproterenol (●), or buffer only (○) for 15 min at 25°, and then for the times indicated with 100 $\mu$g $\alpha$-chymotrypsin/mg of membrane protein. Samples were then removed, washed, and assayed for beta-receptors with 10 nM (−)-[$^3$H]dihydroalprenolol (DHA). Control membranes bound 0.211 ± 0.017 pmole DHA/mg protein.

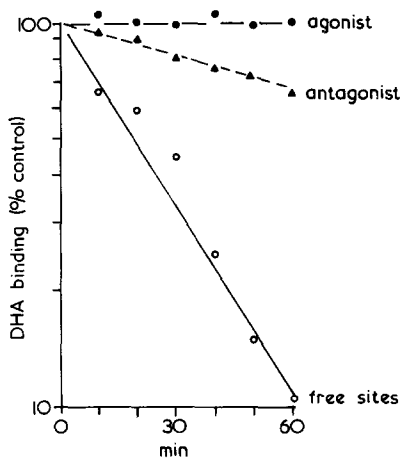


Fig. 2. Effect of heat treatment on beta-receptors. Cardiac membranes (3 mg protein/ml) were incubated for 15 min at 25° in buffer containing 20 $\mu$M (±)-alprenolol (▲), 50 $\mu$M (−)-isoproterenol and 0.1% ascorbate (●), or buffer alone (○). The suspensions were then incubated at 50°. At the times indicated, samples were removed, washed, and assayed for beta-receptors with 10 nM DHA. Control membranes bound 0.231 ± 0.014 pmole DHA/mg protein.

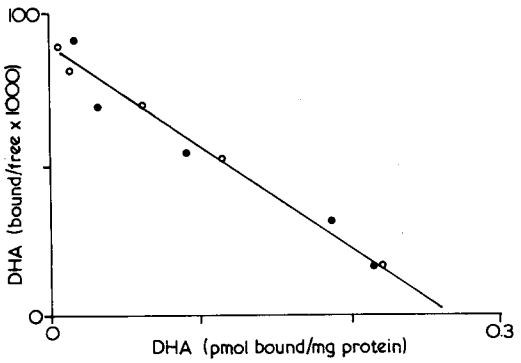


Fig. 3. Binding of (−)-[$^3$H]dihydroalprenolol to heat-treated cardiac membranes. Cardiac membranes (3 mg protein/ml) were preincubated for 20 min at 25° in buffer containing 50 μM (−)-isoproterenol and 0.1% ascorbic acid. The suspension was then incubated for 60 min at 50° and assayed for beta-receptors. Key: specific DHA binding in control (○) and (●) heat-treated membranes.

32 percent in the presence of alprenolol. Remarkably, there was no receptor loss during incubation with 50 mM (−)-isoproterenol and 0.1% ascorbic acid. By comparison, incubation at 50° with 0.1% ascorbic acid alone showed the same rate of beta-receptor loss as for the unoccupied state, and incubation with 50 μM (+)-isoproterenol and ascorbic acid yielded 75 percent loss of receptors (not shown).

*Characteristics of heat-treated beta-receptors.* Scatchard analysis of specific [$^3$H]dihydroalprenolol binding to cardiac membranes heated at 50° for 60 min in the presence of 50 μM (−)-isoproterenol and 0.1% ascorbic acid showed the same maximum binding of 0.27 pmole/mg protein and $K_D$ of 3.8 nM, as in untreated membranes (Fig. 3). Also, as in untreated membranes [11], (−)-isoproterenol was two orders

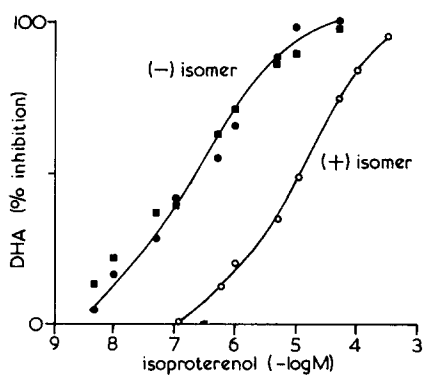


Fig. 4. Inhibition of DHA binding to heat-treated membranes by (+)- and (−)-isoproterenol. Cardiac membranes (3 mg protein/ml) were preincubated for 20 min at 25° in buffer containing 50 μM (−)-isoproterenol and 0.1% ascorbate. The suspension was then incubated for 60 min at 50°, and the membranes were washed and assayed for specific DHA binding with 10 nM DHA and the indicated concentration of (−)-isoproterenol (●) or (+)-isoproterenol (○). Control DHA binding was 0.198 ± 0.011 pmole/mg protein. Also shown are mean control values for (−)-isoproterenol from three other experiments in which membranes were not heated (■).

of magnitude more potent than (+)-isoproterenol in competing with 10 nM (−)-[$^3$H]dihydroalprenolol (Fig. 4). Low concentrations of (−)-isoproterenol were slightly less potent in inhibiting the binding of DHA to heated than to control membranes (Fig. 4).

## DISCUSSION

It is clear from the present results that beta-adrenoreceptors in cardiac membranes are markedly protected from inactivation by heat by isoproterenol and by alprenolol, and that the same ligands significantly facilitate degradation of the receptor protein by α-chymotrypsin. These effects of alprenolol could, in theory, be due to dissolution of the drug in membranes [13] in the region of receptors, rather than to specific binding, although the low concentration of the agent used (20 μM) makes this unlikely. Isoproterenol has a much lower partition coefficient [13] in membranes than alprenolol, was also used at a relatively low concentration (50 mM), and the (−)-isomer was more protective than the (+)-isomer. We conclude that the effects seen on receptor stability are in all probability due to direct binding of these ligands to the receptor protein itself.

Antagonists like alprenolol do not lead to an active conformation of beta-receptors, nor do they lead to receptor desensitization [3, 14]. The potent effects of alprenolol on receptor stability suggest, therefore, that simple binding of this agent is sufficient to induce or stabilize a receptor conformation which is significantly different from active and desensitized conformations, as well as from the resting state. Obviously agonists may also bind to such an intermediate form, before active and desensitized states are produced.

In the present experiments no attempt was made to distinguish between the stability of active and desensitized receptors; it is probable that 50 μM (−)-isoproterenol produces considerable desensitization. Whatever the degree of desensitization, no change in the number of binding sites was observed in the present experiments when cardiac membranes were incubated for 1 hr with 50 μM isoproterenol at either 25° or 50°. In contrast, Mickey *et al.* [3, 15] reported 50 percent reduction of the binding of (−)-[$^3$H]dihydroalprenolol after incubating erythrocytes in 100 μM (−)-isoproterenol at 23° for 3 or more hours.

The receptor changes induced by ligand binding are not known. However, the following information suggests that greater protrusion of the receptor protein from the membrane surface may occur with both agonists and antagonists. The increased activity of α-chymotrypsin in the presence of isoproterenol or alprenolol indicates that receptor peptides sensitive to the enzyme are more exposed to the enzyme. Certainly ligand–receptor complexes did not "dive" beneath the membrane surface in our experiments, even though agonist–receptor complexes are known to interact with a nucleotide binding protein near the cytoplasmic surface [16, 17]. Enzymes bound to hydrophilic supports (to stimulate the membrane environment) are more thermostable than enzymes bound to hydrophobic supports [18]. By analogy, the receptor protein may be more thermostable in

the presence of ligands because of greater protrusion into an aqueous environment. The molecular mechanisms involved in conformational changes are also unknown, although it has been suggested that thiol groups are involved [5, 19, 20].

(−)-Isoproterenol, like other beta-agonists having high efficacy, binds with higher affinity to receptors which are coupled to a nucleotide binding protein than those which are not [17]. Such coupling must modify the conformation of the receptor protein and thus its stability. Since our membranes show activation of adenylate cyclase activity in the presence of (−)-isoproterenol and guanyl-5′-yl imidodiphosphate [10], and since coupling is facilitated by agonists in the absence of the nucleotide [17], near maximum amounts of (−)-isoproterenol–receptor–nucleotide protein complexes were probably present at the outset of our experiments. The effects of such coupling are under study with a different membrane preparation. For the moment it may be noted that heat tends to diminish the highest affinity agonist states (Fig. 4).

Stabilization of the beta receptor protein in the presence of its ligands may have practical applications. For example we have found that cardiac membranes can be stored at 4° in 50 $\mu$M (−)-isoproterenol for a week with negligible losses, whereas half of the binding sites are usually lost in a few days without the agonist. Heat-stabilized protein has the same $K_D$ value for (−)-[$^3$H]dihydroalprenolol after the removal of isoproterenol (Fig. 3).

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