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SPECIFIC AND UNSPECIFIC BINDING OF [^{3}H](−)DIHYDROALPRENOLOL TO CARDIAC TISSUE*

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Abstract—Identification of β-adrenergic receptors has been successfully performed by [^{3}H](−)dihydroalprenolol binding to several tissues of a variety of species. Specific binding sites (receptors) have usually been defined by criteria as: velocity, reversibility, saturability and stereospecificity of binding. However, [^{3}H](−)dihydroalprenolol does not only bind to specific sites but also to unspecific ones. The given definitions of unspecific binding sites are somewhat confusing.

[^{3}H](−)Dihydroalprenolol bound (2670 c.p.m./mg prot.) to cardiac membranes is inhibited in the presence of unlabeled alprenolol in a concentration-dependent manner up to 10^{-3} M. From 10^{-9} to 10^{-5} M this inhibition caused by (−) and (+) alprenolol was found to be stereospecific. In higher concentrations the measured inhibition of [^{3}H](−)dihydroalprenolol binding to its receptor was no longer stereospecific. In heat denatured membranes [^{3}H](−)dihydroalprenolol bound (1335 c.p.m./mg prot.) was identical with that of native membranes in the presence of 10^{-5} M (−) or (+) alprenolol. There was no inhibition of binding, however, in the presence of unlabeled (−) or (+) alprenolol in a concentration range below 10^{-4} M. At higher concentrations there was inhibition of binding, but it was not stereospecific. When an equilibrium of [^{3}H](−)dihydroalprenolol binding to all cardiac membranes (native or heat denatured) had been established, addition of the unlabeled ligand led to a concentration-dependent and rapid displacement of the labeled ligand. Again, stereospecificity of the displacement was only observed in the native membranes. Inhibition of adenylate cyclase activity was measured at the same (−) and (+) alprenolol concentrations and showed the same stereospecific effects.

Thus, the results indicate that the loss of stereospecificity in heat denatured membranes and in native membranes correlates with unspecific binding of [^{3}H](−)dihydroalprenolol to cardiac membranes. It is quantitatively identical. It is concluded, that out of the criteria for specific binding mentioned, only stereospecificity seems to be valid for [^{3}H](−)dihydroalprenolol binding to the β-adrenergic receptor.

Receptors have formerly been defined rather indirectly by observations and calculations of certain physiological and pharmacological effects after addition of hormones or drugs [1]. It should be kept in mind, however, that the term "receptor" is only a general one and its use implies a lack of knowledge of chemical structures involved in the interactions. Thus, a hormone receptor is supposed to be a bifunctional element that, in response to its interactions with the hormone, leads to the generation of a stimulus which in turn triggers some kind of a measurable response [2]. In the last few years there has been a great interest in characterizing receptors with labeled hormones and drugs by binding studies [3–9]. This way, it has become possible to note details of drug–(hormone)–receptor interactions, the initial step of an effect of a pharmacon.

Labeled drugs and hormones, however, do not bind to their respective receptor only. Several investigators have reported experiments demonstrating binding of radioactively labeled insulin to, for instance, talc, silicon, or glass tubes [10] or of labeled nerve growth factor to glass beads [11]. Although this binding showed properties very similar to that of specific hormone–receptor binding (i.e. high affinity, time and concentration-dependent binding, displacement by unlabeled hormones etc.) it must be termed as unspecific binding, because there is no biological activity as an answer to this "hormone–receptor binding" [12].

Recently a great number of reports have demonstrated binding of [^{3}H](−)dihydroalprenolol to a variety of cell membranes [13–19]. The specific binding of this β-adrenergic receptor blocking agent to the membrane bound β-receptor causes an alteration of the activity of the receptor-coupled adenylate cyclase [5]. There is, however, no generally accepted definition of "specific" and "unspecific" binding of this drug to its receptor. There are reports terming that binding of [^{3}H](−)dihydroalprenolol to membranes as "unspecific" which cannot be inhibited in the presence of unlabeled β-blocking agents in concentrations greater than 10^{-6} M (−) alprenolol [20], 10^{-6} M (−) propranolol or (−) alprenolol [21], 10^{-5} M (±) propranolol [22–23], 10^{-4} M (±) propranolol [24], 10^{-5} M (−) propranolol or (−) alprenolol [25], or 10^{-5} M (−) isoprenaline [26]. The reason for this definition is not quite clear either, and "sometimes rather confusing" [27] as "unspecific" binding of this labeled drug to cell membranes may amount to 3–50 per cent of total binding [24–26].

Thus, there is a need for an exact determination of specific and unspecific [^{3}H](−)dihydroalprenolol binding to biological material.

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MATERIALS AND METHODS

Materials. All nucleotides, creatine phosphate and creatine kinase were from Boehringer, Mannheim. [^{3}H](−)dihydroalprenolol (sp. act. 32 Ci/m-mole) was from NEN, Dreieichenhain, Germany. The following compounds were generous gifts from Hässle, Göteborg, Sweden: (−) alprenolol, (+) alprenolol.

Preparation of membrane particles. Male guinea-pigs (250–350 g) received i.m. injections of reserpine (3 mg/kg) 24 and 12 hr before decapitation. Hearts were quickly removed, freed from connective tissue, atria and valves; the ventricles were disected and washed free from blood. The washed ventricular tissue was minced and a crude membrane fraction was prepared by a modification [9] of the method described by Drummond and Severnson [28].

Adenylate cyclase assay. A two step incubation was employed for concentration–response curves. During the first step (15 min at 0°) myocardial membranes were preincubated in the absence of adenylate cyclase reagents with additions (agonists, antagonists, and Gpp(NH)p)*; adenylate cyclase reagents were added at the end of the preincubation and during the second step incubated at 37° for 15 min. The final concentration of reagents was: $MgCl_2$, 1 mM; creatin phosphate, 8.7 mM; creatin kinase, 50 U/ml; AMP, 1 mM; isobutylmethylxanthine, 5.4 mM; Tris–HCl buffer, pH 7.8, 50.5 mM; [^{32}P]ATP, 0.3 mM (25–50 c.p.m./pmole). The final reaction volume was 60 μl and the concentration of myocardial membrane was kept between 80–160 μg prot./60 μl. The reaction was terminated and [^{32}P]cAMP was isolated as described by Salomon *et al.* [29]. Duplicates agreed within 7 per cent. The activity of adenylate cyclase is expressed in nmoles cAMP produced per mg of protein per 10 min.

Binding assay. Membrane suspensions, freshly prepared (0.4–0.8 mg of protein) were incubated at 37° in 50 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$ with labeled β-receptor antagonists and various additions (final vol., 1 ml). At the given time (usually after 15 min) membrane bound and free ligand were separated by rapid filtration followed by two washes (10 ml each) on Whatman fiberglass filters (GF/C). Radioactivity on filters was determined by liquid scintillation counting [8].

Calculations. K_i values for β-receptor antagonists were calculated using equations given by Dixon and Webb [30]. Different concentrations of the antagonist were tested in the presence of a constant concentration of an agonist and linear regression analysis was performed after transformation of the data [9] into

$$\frac{V-V_i}{V} = \frac{I}{K_i} \cdot \frac{K_a}{K_a + A}$$

where V is the response of adenylate cyclase in the presence of the agonist alone at concentration (A) with half-maximum response at the concentration K_a; V_i is the response in the presence of the β-receptor antagonist at the concentration I, and K_i is the concentration of the antagonist leading to 50 per cent occupancy of the receptor.

* The abbreviations used are: Gpp(NH)p, 5′-guanylyl-imidodiphosphate; cAMP, cyclic adenosine 3′,5′-monophosphate.

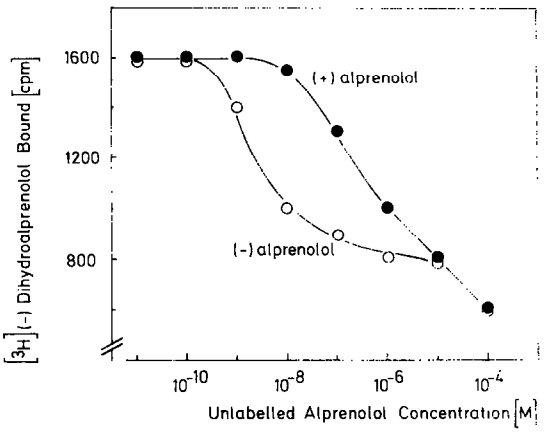

Fig. 1. Concentration-dependent inhibition of [^{3}H](−) dihydroalprenolol binding to crude cardiac cell membranes by (−) and (+) alprenolol. The incubation medium (1 ml) contains freshly prepared membranes (0.6 mg prot.), [^{3}H](−)dihydroalprenolol 1.6×10^{-9} moles/ml, 5.0 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$ and increasing concentrations of unlabeled (−) and (+) alprenolol. After 15 min at 37°, incubation was stopped by rapid filtration. Values are means from triplicate experiments.

All experiments were performed in duplicate assays and at least twice. Usually the results are given as means ± S.E.M.

RESULTS

[^{3}H](−)dihydroalprenolol and increasing concentrations of unlabeled (−) and (+) alprenolol are incubated together with cardiac membranes of guinea-pigs and the inhibition of labeled alprenolol binding to its binding sites is measured (Fig. 1). The effect of β-adrenergic

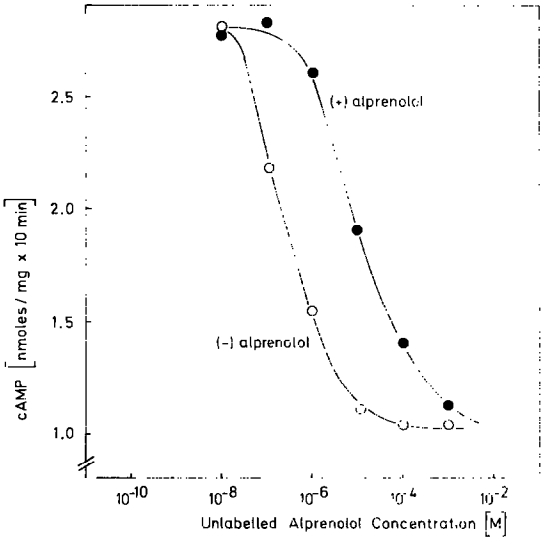

Fig. 2. Concentration-dependent inhibition of Gpp(NH)p/isoprenaline stimulated guinea-pig myocardial adenylate cyclase. Isoprenaline concentration was 12.7 μM and Gpp(NH)p 7 μM. The same membrane preparation was used as in Fig. 1. Calculations (see Methods) result in corresponding K_I and K_D values (10^{-9} M). Means of triplicate experiments.

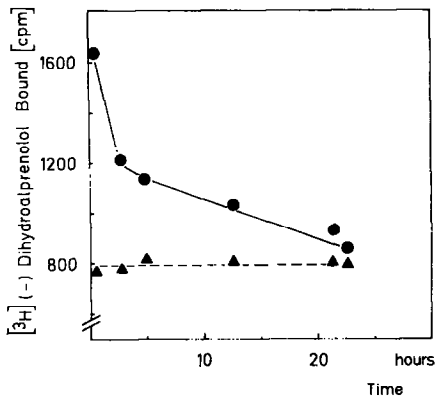

Fig. 3. [^{3}H](−)Dihydroalprenolol binding to cardiac membranes during prolonged incubation at 37°. The cardiac membranes are incubated at distinct intervals (●——●) with [^{3}H](−)dihydroalprenolol (1.6×10^{-12} moles/ml) and in parallel series with additional 10^{-5} M (−) alprenolol (▲——▲). The [^{3}H](−)dihydroalprenolol binding capacity decreases steadily, reaching that of "unspecific binding" after 23 hr of incubation. Means of duplicate experiments.

antagonists on the adenylate cyclase activity was measured by the concentrations of cAMP generated in the same membrane preparation. Concentration–response curves demonstrate a 100 fold greater potency of the (−) isomers as compared with the (+) isomers in inhibiting the binding of [^{3}H](−)dihydroalprenolol or in inhibiting the activated adenylate cyclase (Fig. 2).

The calculated K_i (K_i = inhibitor constant for the adenylate cyclase activity and K_D values (K_D = dissociation constant of the drug–receptor complex, see Calculations) are identical: $K_i = K_D = 10^{-9}$ M [9]. These experiments demonstrate furthermore that the plots of the action of the (−) and (+) isomers of the β-adrenergic antagonists intersect at a concentration of 10^{-5} M: greater concentrations of (−) and (+) isomers do not lead to stereospecific inhibition of binding any more.

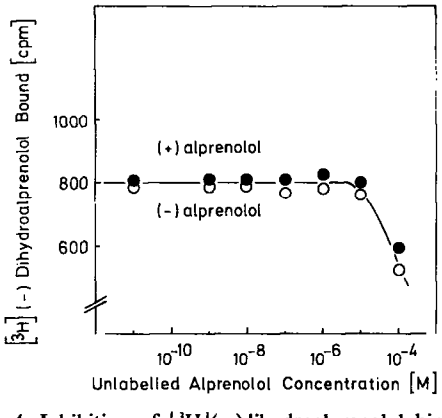

Fig. 4. Inhibition of [^{3}H](−)dihydroalprenolol binding to cardiac cell membranes by (−) and (+) alprenolol. The cardiac cell membranes are heated for 23 hr at 37°, before incubation with [3H](−)dihydroalprenolol and increasing concentrations of (−) and (+) alprenolol are performed. At concentrations of 10^{-9}–10^{-5} M of (−) or (+) alprenolol there is no stereospecific inhibition of binding at all, at greater concentrations than 10^{-5} M a non-stereospecific effect results. Means of duplicate experiments.

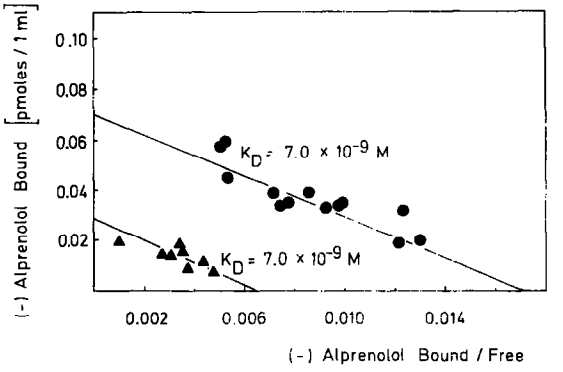

Fig. 5. Loss of specific [^{3}H](−)dihydroalprenolol binding sites after 8 hr of incubating membranes at 37°. Freshly prepared membranes are incubated for 15 min at 37° with [^{3}H](−)dihydroalprenolol (1.6×10^{-12} moles/ml) and increasing concentration of (−) alprenolol. The result is plotted according to Scatchard [31], the dissociation constant K_D is calculated from the slope of the plot $K_D = 7.0 \times 10^{-9}$ M and the maximal binding sites are calculated from the intercept on the ordinate (0.15 pmoles/mg protein). The same experiments performed with 8 hr-heated cardiac cell membrane at 37° and the data plotted in the same way resulted in $K_D = 7.0 \times 10^{-9}$ M and binding capacity of 0.06 pmoles/mg protein. There is no change of receptor affinity for alprenolol but a loss of binding sites.

Cardiac membranes were heated at 37° over 23 hr (Fig. 3) in order to distinguish and get further information on two apparent types of binding sites (one type of binding site where there is stereospecific inhibition of binding—obvious in the presence of concentrations $< 10^{-5}$ M—and one type of binding site where there is inhibition of binding of [^{3}H](−)dihydroalprenolol by alprenolol, but no stereospecificity—obvious with concentrations $> 10^{-5}$ M). At definite intervals these pretreated membranes were incubated with [^{3}H](−)dihydroalprenolol and in additional series with 10^{-5} M (−) alprenolol. At these conditions the maximal binding of [^{3}H](−)dihydroalprenolol diminishes distinctly, but the binding capacity remaining after addition of 10^{-5} M (−) alprenolol stays the same throughout the 23 hr. Binding experiments with the (+) and (−) isomers of alprenolol with freshly prepared cardiac membraines are demonstrated in Fig. 1. The same experiments performed with membranes heated at 37° for 23 hr (Fig. 4) do not show any alteration of binding in the presence of increasing concentrations up to 10^{-5} M. Concentrations of unlabeled β-blocking drugs greater than 10^{-5} M are able to inhibit binding of [^{3}H](−)dihydroalprenolol but without stereospecific effect. About 50 per cent of the total bound [^{3}H](−)dihydroalprenolol is inhibited to bind in such a manner. This amount is therefore termed "unspecific binding".

Heating of cardiac cell membranes might either destroy the membrane proteins (receptors) or change the affinity of specific binding sites or both. Figure 5 demonstrates the result of equilibrium binding of [^{3}H](−)dihydroalprenolol and increasing concentrations of unlabeled alprenolol on freshly prepared cardiac membranes. The values plotted according to Scatchard [31] show a linear plot after subtraction of the amount of labeled alprenolol bound in the presence of

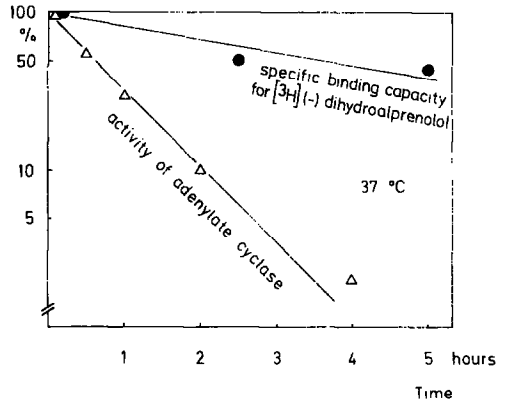

Fig. 6. Time-dependent loss of specific myocardial binding sites and activity of myocardial adenylate cyclase. At indicated times (●——●) heated cardiac cell membranes at 37° are incubated with [^{3}H](−)dihydroalprenolol ($1.6 \cdot 10^{-12}$ pmoles/ml) for 15 min. Binding to freshly prepared membranes indicates 100 per cent of specific binding sites, after subtraction of the amount of [3H](−)dihydroalprenolol bound at the presence of 10^{-5} M (−) alprenolol. In the same preparation as used above (heated at 37°) stimulated adenylate cyclase activity, △—— △, (100 per cent = in the presence of isoprenaline 12.7 μM and Gpp(NH)p 7 μM) is measured.

10^{-5} M(−) alprenolol. The disociation constant (K_{II}) calculated from the slope of the plot is about 7×10^{-9} M. The intercept with the ordinate gives the maximal number of receptor sites (0.07 pmoles/ml = 0.15 pmoles/mg prot.). The same experiment was performed after heating the membranes for 8 hr. After plotting the results in the same way, a parallel slope indicates an identical dissociation constant (K_{II} · 7×10^{-9} M). The intercept of this slope, however, indicates a distinctly smaller number of receptor sites (0.06 pmoles/mg prot.).

The Gpp(NH)p and isoprenaline stimulated adenylate cyclase of the same cardiac cell membranes heated at 37° was also measured at different time intervals (Fig. 6). The activity of the adenylate cyclase declined rapidly (50 per cent in 30 min) whereas the loss of [^{3}H](−)dihydroalprenolol binding capacity is apparently slower (50 per cent in 5 hr).

The [^{3}H](−)dihydroalprenolol binding experiments so far in fact have shown that unlabeled ligands at increasing concentration do inhibit the binding of the labeled ligand to specific and unspecific binding sites (Figs. 1 and 4). In order to demonstrate that the unlabeled ligand does actually displace the [^{3}H](−)dihydroalprenolol from both specific and unspecific binding sites, unlabeled (+) and (−) alprenolol

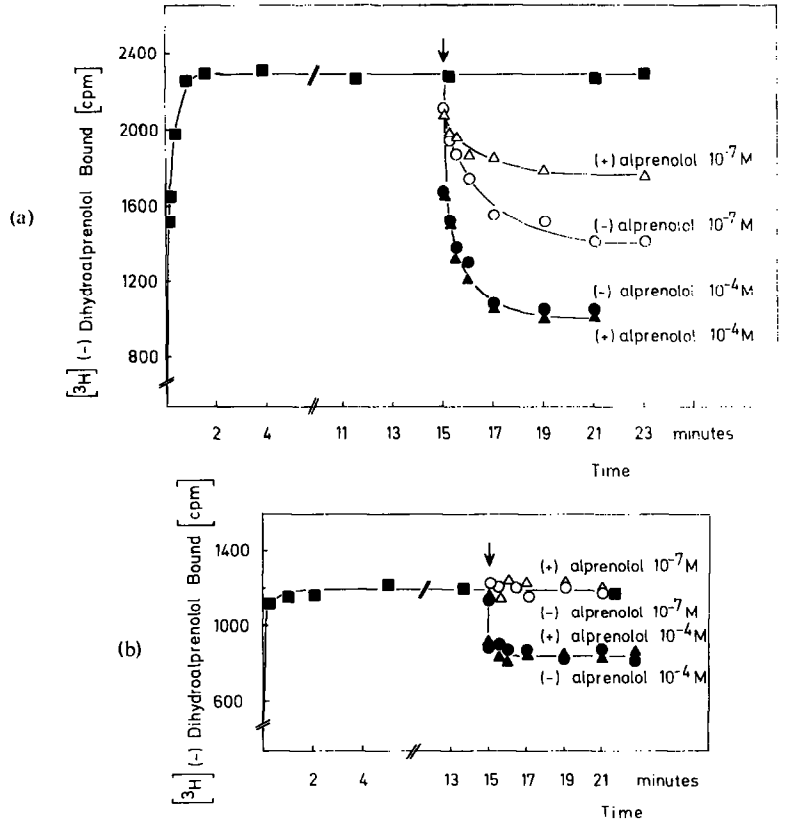

Fig. 7. Displacement of bound [^{3}H](−)dihydroalprenolol from cardiac cell membranes. (a) Freshly prepared membranes were incubated at 37° with [^{3}H](−)dihydroalprenolol (1.6×10^{-12} moles/ml) for 15 min (until equilibrium), then (↓) two different concentrations of (−) and (+) alprenolol were added: △—△ (+) alprenolol 10^{-7} M, ○——○ (−) alprenolol 10^{-7} M, ▲——▲ (+) alprenolol 10^{-4} M, ●——● (−) alprenolol 10^{-4} M. At the indicated time intervals the reaction was stopped by rapid filtration. [^{3}H](−)Dihydroalprenolol is displaced in a concentration dependent and stereospecific manner. Means of triplicate experiments. (b) Same experiment as above, but with heated membranes (23 hr at 37°). There is no stereospecific displacement of [^{3}H](−)dihydroalprenolol binding in these membranes at all, although the unlabeled ligand does displace the bound labeled ligand at high concentrations (10^{-1} M).

were added after a binding equilibrium had been established (Fig. 7).

In freshly prepared cardiac membranes the addition of (+) and (−) alprenolol (10^{-7} M) leads to a clearly stereospecific and rapid displacement of the labeled ligand. Addition of (+) and (−) alprenolol at higher concentration (10^{-4} M), though causing a greater displacement, does not lead to a stereospecific displacement of [^{3}H](−)dihydroalprenolol (Fig. 7a). In heat denatured cardiac membranes the unlabeled ligand does not displace [^{3}H](−)dihydroalprenolol in low concentrations (10^{-7} M). There is no stereospecific effect at high concentrations (10^{-4} M), which do displace the labeled ligand quite as rapidly, as demonstrated in Fig. 7b.

DISCUSSION

Hormone or drug actions are usually preceded by an interaction with their specific receptors [32]. Therefore, this specific drug–receptor interaction has caught much interest in the past few years when it became possible to measure this first step of drug action with radioactively labeled substances. Labeled drugs do not, however, bind only to their specific receptors [2, 4, 5, 20, 22, 27]. A definition of "unspecific" binding is, therefore, essential for the evaluation of experimental binding data. Some criteria have been established to identify specific β-receptors in cell membrane preparations: binding had to be rapid, reversible, saturable and stereospecific, and it has to exhibit high affinity for β-adrenergic agents [17, 33–35]. In this respect [^{3}H](−)dihydroalprenolol binding to cardiac cell membranes might be termed "specific" as it is very rapid (completed within less than 4 min [9]), reversible and stereospecific (Fig. 1) [25]. However, unspecific bindings to these membranes may also be reversible, as shown in Fig. 7a and b, where [^{3}H](−)dihydroalprenolol binding to heat-denatured membranes could be displaced easily by unlabeled alprenolol in concentrations above 10^{-5} M. Thus, reversibility of drug–receptor binding is not necessarily a criterion for specificity of binding to biological material [36]. The experiments represented in Figs. 1 and 4 do, however, show that there is no stereospecificity of the effect at the high alprenolol concentrations ($\geqslant 10^{-5}$ M). Under our experimental conditions the amount of bound [^{3}H](−)dihydroalprenolol is identical:

1. when [^{3}H](−)dihydroalprenolol is bound in the presence of unlabeled (−) or (+) alprenolol in a concentration of 10^{-5} M in native cardiac membranes (Fig. 1);

2. in the presence of that unlabeled alprenolol concentrations where the experimental curves for the (−) and (+) isomers meet (where there is no stereospecific effect at higher concentrations) (Fig. 1);

3. in the presence of 10^{-5} M unlabeled (−) alprenolol to heated membranes (Fig. 3);

4. in the presence of 10^{-9}–10^{-5} M unlabeled (−) or (+) alprenolol to heat-denaturated membranes (Fig. 4).

We, therefore, think that this value gives us the true amount of unspecific [^{3}H](−)dihydroalprenolol binding. This hypothesis is supported by the experiments shown in Fig. 2, demonstrating that higher concentrations than 10^{-3} M alprenolol do not further inhibit the adenylate cyclase activity and do not show stereospecificity, either. This point of intersection (concentration of (−) and (+) alprenolol 10^{-3} M) correlates with the concentration of unlabeled 10^{-5} M (−) and (+) alprenolol in the receptor binding studies. The concentration differences (10^{-5} M and 10^{-3} M) are caused by the stimulation of adenylate cyclase by isoprenaline (10^{-6} M) and Gpp(NH)p (10^{-5} M), which shift the dose–response curves of the adenylate cyclase to higher concentrations. This is a known phenomenon [34, 35]. Calculations [30] of the K_h and K_l of alprenolol from our experimental data lead to identical constants independent of the isoprenaline and Gpp(NH)p stimulation ($K_h = K_l = 10^{-9}$ M). If we had used a distinct low isoprenaline concentration and thus had stimulated the adenylate cyclase activity less, we would have made the assay more sensitive for the inhibition by β-receptor blocking agents. These experimental differences do not appear when the K_l-value is calculated.

In the time course of the membrane alteration during incubation at 37°, adenylate cyclase activity and [^{3}H](−)dihydroalprenolol binding capacity decrease. The Scatchard plots of the binding data with freshly prepared membranes and with 8 hr-heated membranes indicate a loss of binding sites rather than an affinity change of the receptors. It is quite interesting to note that the amount of "unspecific" binding stays the same throughout the whole inactivation period (Fig. 3).

In contrast to our results, Harden *et al.* [37] and Caron and Lefkowitz [38] found no further inhibition of binding of labeled alprenolol at concentrations of unlabeled β-blocking agents $> 10^{-5}$ M. They did not give the experimental data, however. Cuatrecasas and Hollenberger [10] demonstrated displacement of several labeled hormones from nonbiological material by unlabeled hormones. They concluded, too, that reversibility of binding certainly cannot be used as a proof for specificity of hormone–receptor binding.

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