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EVIDENCE FOR NEGATIVE COOPERATIVITY AMONG β-ADRENERGIC RECEPTORS IN CARDIAC AND LUNG TISSUE OF GUINEA PIG*

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Abstract— Stereospecific binding sites for (−)-[^{3}H]dihydroalprenolol, a β-adrenergic antagonist, have been identified in guinea-pig myocardial cell membranes and lung tissue preparations. A close correlation between the abilities of β-adrenergic antagonists to compete with labelled antagonist binding and to block the response of isoproterenol plus Gpp(NH)p† stimulated adenylate cyclase has been found. Equilibrium binding of (−)-[^{3}H]dihydroalprenolol to binding sites of cardiac and lung tissue displays characteristics consistent with negative cooperativity among the DHA binding sites: Scatchard plots are curvilinear with upward concavity. The existence of site–site interactions of the negatively cooperative type was demonstrated directly by the ability of unlabelled (−)alprenolol to accelerate the dissociation of (−)-[^{3}H]dihydroalprenolol under conditions where rebinding of the radioligand does not occur.

The pharmacological and physiological effects of β-adrenergic receptor stimulation are consequences of drug receptor interactions at the molecular level.

By the introduction of labelled β-adrenergic antagonists it has become possible to characterize the β-adrenergic receptor directly and to demonstrate its close connection with the adenylate cyclase [1–4]. In general these characteristics include the appropriate affinity, reversibility, specificity of binding and stereospecificity as well as saturability of the binding sites. Furthermore, a recently observed site–site interaction among membrane bound receptors has caught the general interest. Limbird and Lefkowitz [5] showed, by analysis of steady-state data for (−)-[^{3}H]dihydroalprenolol binding to frog erythrocyte membranes, the existence of negative cooperativity, meaning a concentration dependent site–site interaction causing decreasing affinity of the receptor for the drug at high drug or hormone concentrations.

The dissociation of ^{125}I-insulin from its receptor (in human lymphocytes culture) in the absence and presence of unlabelled insulin followed different kinetics. This has been taken as evidence for site–site interactions among insulin receptors, because the presence of unlabelled hormone increased the dissociation rate [6]. These changes in the states of insulin receptors might explain several pathophysiological conditions [7].

It is the purpose of this study to examine the existence of site–site interactions among DHA binding sites in mammalian cardiac and lung tissue.

MATERIALS AND METHODS

Materials. The chemicals used to assay adenylate cyclase were of analytical purity and were purchased from E. Merck, Darmstadt, Germany and Boehringer-Mannheim, Germany. (−)-[^{3}H]dihydroalprenolol (sp. act. 32 Ci/mmole) was from NEN, Dreieichenhain, Germany.

The following compounds were generous gifts from the respective companies: (−)-propranolol, (+)propranolol, (±)propranolol, (ICI-Pharma, Plankstadt, Germany); (−)oxprenolol, (+)oxprenolol, (±)oxprenolol (Ciba-Geigy, Wehr, Germany); (−)alprenolol, (+)alprenolol, (±)alprenolol (Astra Chemicals, Wedel, Germany).

Preparation of a crude membrane fraction. Male guinea-pigs (250–350 g) were decapitated; their hearts were quickly removed, and freed from connective tissue, atria and valves. The ventricles were dissected and washed free from blood. The washed ventricular tissue was minced and a crude membrane fraction was prepared as described by Drummond and Severson [8]. A crude membrane fraction of lung tissue was prepared in the same way.

For the adenylate cyclase assay the membranes were quickly frozen and stored at −40°. Protein was determined by the method of Lowry *et al.* [9].

Binding assay. Membrane suspensions, freshly prepared (0.4–0.8 mg protein) were incubated at 37° in 7.5 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$ with labelled β-receptor antagonists and various additions (final vol: 1 ml). At the given time (usually 15 min) membrane bound and free ligands 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 (Insta Fluor, Fa. Packard).

Unspecific binding was determined in the presence of unlabelled (−) and (+)alprenolol, 10^{-5} M. It amounted to about 50 per cent of maximal binding, as described by Krawietz and Erdmann [10].

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

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† Abbreviations used are: Gpp(NH)p, 5′-guanylylimidodiphosphate; DHA, dihydroalprenolol; cAMP, cyclic adenosine 3′, 5′ monophosphate; STH, somatotropic hormone.

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; creatinphosphate, 8.7 mM; creatinkinase, 50 U/ml; cyclic 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 and 160 μg protein/60 μl. The reaction was terminated and [^{32}P]cAMP was isolated as described by Salomon *et al.* [11]. Duplicates agreed within 7 per cent. The activity of adenylate cyclase is expressed in pmoles cAMP produced per mg of protein per 10 min.

Dissociation kinetics. For these experiments the radioactive ligand, usually 6.4 nM, was incubated with membranes (8.2 mg protein/ml) for 15 min at 37° or 25°. Aliquots of 100 μl each, containing the usual binding assay, were then transferred to a series of tubes that contained 10 ml of 7.5 mM Tris–HCl and 2.5 mM $MgCl_2$, pH 7.4, with or without 10^{-6} M unlabelled (−)alprenolol at indicated intervals.

Further dilution experiments showed that a dilution beyond 1:60 of the incubation medium was sufficient to prevent reassociation of radioligand. The 100-fold dilution employed would be more than enough. At appropriate intervals, two tubes from each set (with or without unlabelled (−)alprenolol) were vacuum filtered and the radioactivity was measured as described before. Parallel experiments are performed with the same membranes heated for 23 hr at 37°. Binding to membranes prepared in this way has been proved to be unspecific [10]. This 'unspecific binding' was subtracted from the total amount of bound radioactivity in each experiment. The radioactivity bound to the membranes at different times was expressed as a percentage of the radioactivity at $t=0$ (being defined as the time immediately after dilution).

(a) Calculations: K_i values for β-receptor antagonists were calculated using equations given by Dixon and Webb [12]. 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 [14] into

$$\frac{[V - V_i]}{[V_i]} = \frac{[I]}{[K_i]} \times \frac{[K_a]}{[K_a + A]}$$

where V is the response of adenylate cyclase in the presence of the agonist alone at concentration (A) with half-maximal 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.

All experiments were performed in duplicate assays and at least twice. The results are usually given as mean ± S.E.M.

(b) To estimate the amount of binding sites and the dissociation constants, calculations according to Weidemann *et al.* [13] were performed. The data were evaluated as follows: the reaction for binding is $[R] + [L] \rightleftharpoons [RL]$; R= receptor, L= ligand, RL= receptor–ligand complex.

By introducing the dissociation constants $[K_D] = [R][L]/[RL]$ and the total number of binding sites $= R_t$, $[R_t] = [R] + [RL]$, one obtains for the concentration dependence:

$$[RL] = \frac{[R_t] \cdot [L]}{[K_D] + [L]} \quad (1)$$

If there are two different binding sites R_t', R_t'' with two different dissociation constants K_D' and K_D'' one obtains for the concentration dependence of the total binding:

$$[RL] = \frac{[R_t'] \cdot [L]}{[K_D'] + [L]} + \frac{[R_t''] \cdot [L]}{[K_D''] + [L]} \quad (2)$$

In this plot (2) a linear relation should be obtained for a single binding site and a nonlinear relation for two or more binding sites. For the calculations a Hewlett-Packard HP 9830 computer programme was developed, which fitted the experimental values according to the Gauss–Newton iteration.

RESULTS

(−)-[^{3}H]Dihydroalprenolol is used as a tracer for β-adrenergic receptors in cardiac tissue [14] as well as in lung tissue. If increasing concentrations of unlabelled β-adrenergic blocking agents are added to the incubation medium containing 1.5 nM (−)-[^{3}H]dihydroalprenolol and cardiac or lung tissue, a stereospecific inhibition of binding of the labelled β-adrenergic antagonist can be demonstrated by (+) and (−)isomers (Figs. 1a and 2a). The order of efficacies of alprenolol isomers in inhibiting binding of (−)-[^{3}H]dihydroalprenolol to its binding sites in both tissues and those necessary for the inhibition of membrane bound adenylate cyclase activity correlate well, as shown in Figs. 1 and 2. The calculated dissociation constants for (−)alprenolol binding to the receptor (K_D) and the calculated inhibition constants of the enzyme (K_i) are congruent ($K_D = K_i = 10^{-9}$ M) [10, 12, 14].

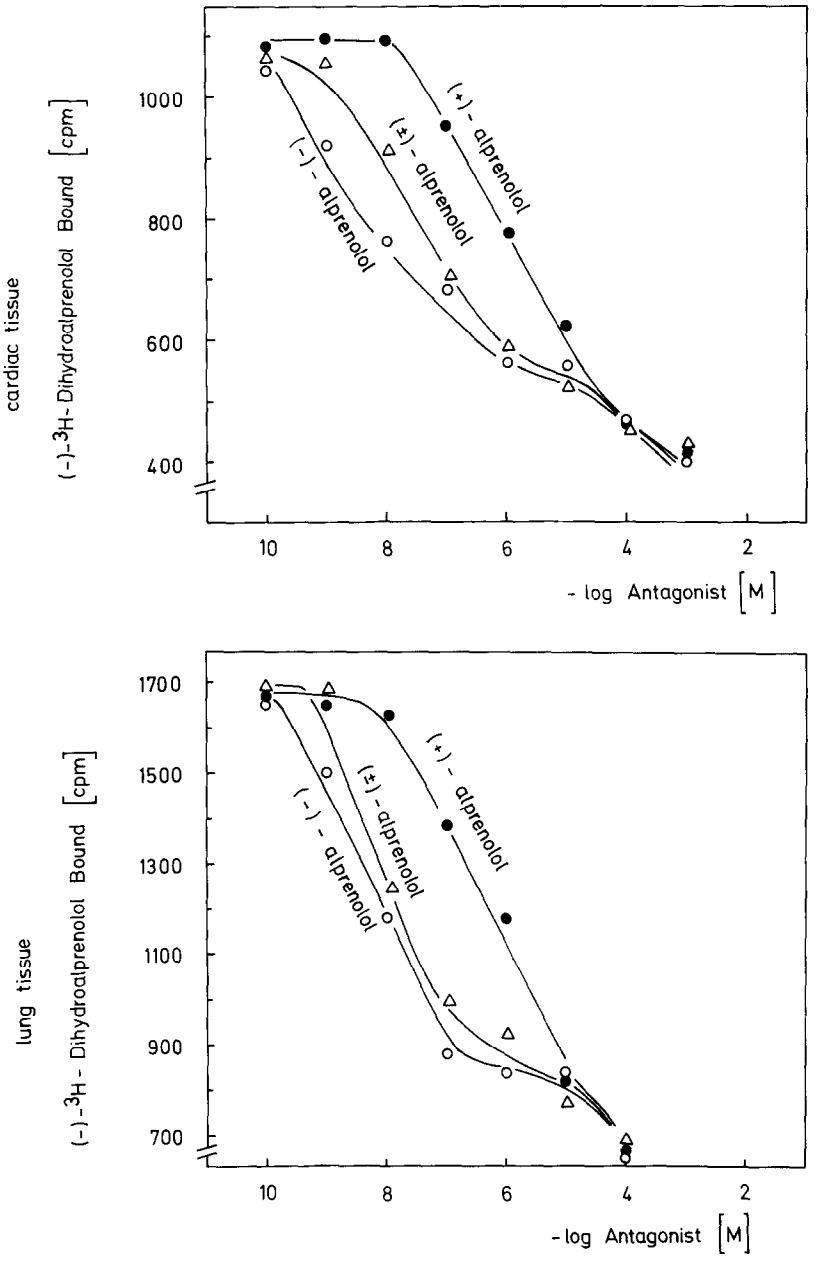
In the experiments depicted in Figs. 1b and 2b the activity of the adenylate cyclase is measured in the presence of β-adrenergic agonist and antagonist together with Gpp(NH)p. Recently, Howlett *et al.* [15] reported that nucleotides decrease the affinity of β-adrenergic agents to the β-adrenergic receptor. These results could be confirmed in our laboratory in cardiac tissue of guinea pig. Therefore, the concentration differences at 50 per cent inhibition of the adenylate cyclase and 50 per cent inhibition of antagonist–receptor binding are caused by the combined stimulation of the adenylate cyclase by Gpp(NH)p and isoproterenol. This phenomenon is caused by Gpp(NH)p and it may be one of the many, but only partly discovered, actions of this nucleotide [16]. The (−)isomers are about 100-fold more potent than the (+)isomers (similar results have been obtained with oxprenolol and propranolol).

To determine the affinity of β-adrenergic antagonists to the binding sites of cardiac and lung tissue, steady-state binding experiments were performed. Labelled alprenolol in a concentration of 1.5 nM was added together with increasing amounts of the respective unlabelled drug to the membranes at 37°. The data obtained from these binding experiments are plotted according to Scatchard [17] (Figs. 3 and 4). These experiments yield plots with upward concavity. With a computer programme (see Materials and Methods) it is

possible to calculate at least two types of alprenolol binding sites. From the two different slopes of the plots two different affinities for the drug may be calculated with two different binding capacities (for data see Figs. 3 and 4 and Table 1). In cardiac membranes (Fig. 3) a specific binding site R_1 with high affinity ($K_D' = 6 \times 10^{-9}$ M) for the drug has only a low binding capacity ($R_1 = 0.18$ pmoles/mg protein), in contrast to R_2 which has a high binding capacity ($R_2 = 22.8$ pmoles/mg protein) and a very low affinity ($K_D'' = 4.3 \times 10^{-6}$ M) in lung tissue (Fig. 4) a similar binding capacity of R_1 binding site ($R_1 = 0.13$ pmoles/mg protein) does exist with a high affinity ($K_D' = 3.3 \times 10^{-9}$ M).

The amount of R_2 binding sites differed in the two organs, however. This may be explained methodically, as evidenced in the Scatchard plot, because a slight deviation of the slope causes a large alteration in the intercept with the ordinate (i.e. the binding capacity [18].

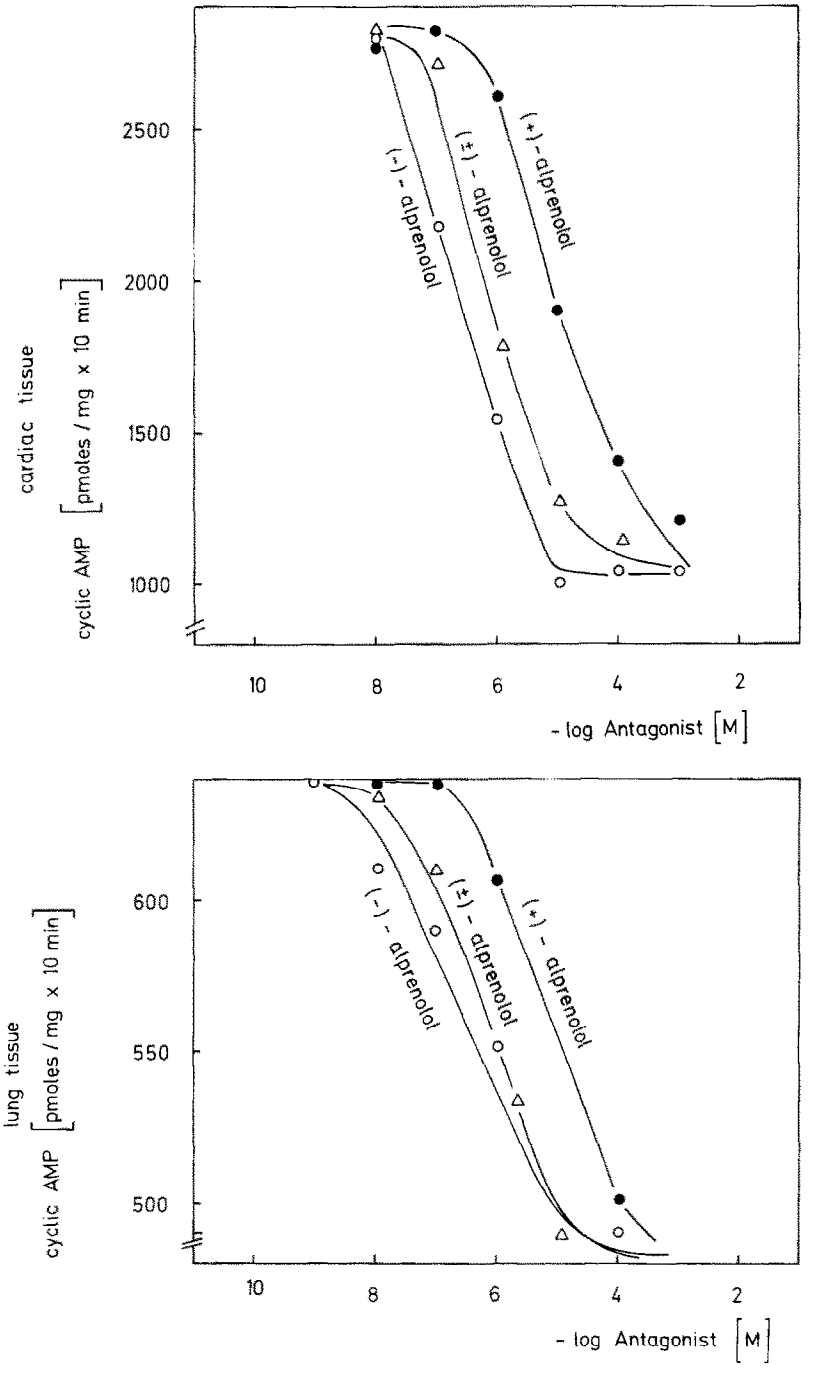
Hill plots of the same binding experiments constantly display slopes (n_H) less than 1(0.5–0.9) in each



Figs. 1a and 2a. Inhibition of (−)-[^{3}H]dihydroalprenolol binding to myocardial and lung tissue by (−)alprenolol (O——O), (+)alprenolol (●——●) and (±)alprenolol (△——△). Membranes (cardiac tissue, lung tissue) were incubated with 7.5 mM Tris–HCl (pH 7.4), 2.5 mM $MgCl_2$ and 1.5 nM (−)-[^{3}H]dihydroalprenolol and the indicated concentrations of unlabelled alprenolol. Means of duplicate experiments.

experiment (Figs. 3 and 4, inset). The Hill plot is assumed to render an index for cooperativity if other explanations can be excluded. The slope (n_H) is less than 1 in cases of negative cooperativity but also in the case of heterogenity of binding sites [19]. It is impossible to discriminate by steady-state binding data between the existence of heterogenous binding sites with distinct affinities and the existence of negatively cooperative site–site interactions.

Therefore, a direct kinetic approach designed by De Meyts [20] was applied in order to look for the existence of negatively cooperative interactions among the



Figs. 1b and 2b. Inhibition of isoproterenol–Gpp(NH)p stimulated adenylate cyclase by the indicated concentrations of alprenolol. Isoproterenol concentration was 12.7 μM and Gpp(NH)p, 7 μM. Basal activity of adenylate cyclase of cardiac tissue (1b) was 594 pmoles/mg × 10 min; maximally stimulated activity was 3540 pmoles/mg × 10 min. Basal activity of adenylate cyclase of lung tissue (2b) was 145 pmoles/mg × 10 min; maximally stimulated activity was 635 pmoles/mg × 10 min. The experimental points are mean values from duplicate experiments.

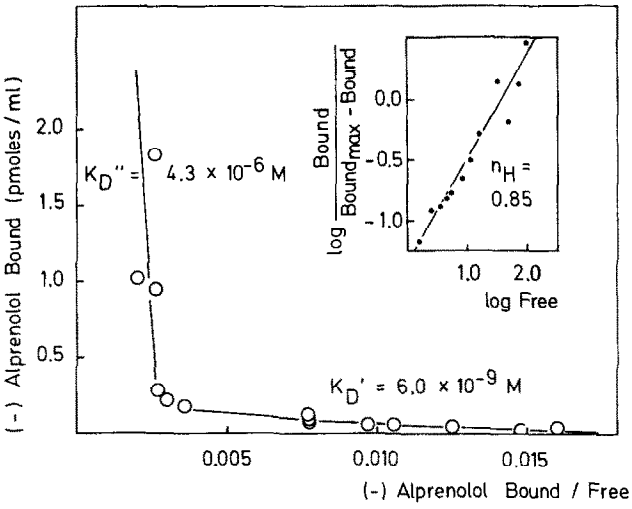


Fig. 3. Equilibrium binding of (−)-[^{3}H]dihydroalprenolol to cardiac membranes. One half milligramme membrane protein was incubated with 1.5 nM (−)-[^{3}H]dihydroalprenolol with increasing concentrations of unlabelled (−)alprenolol at 37° for 15 min. Each value was determined in triplicate. The data are plotted according to Scatchard [17], as explained in Materials and Methods. Inset: the data plotted according to Hill (1910), with a slope of n_H= 0.85. Unspecific binding (as defined in Materials and Methods) is subtracted.

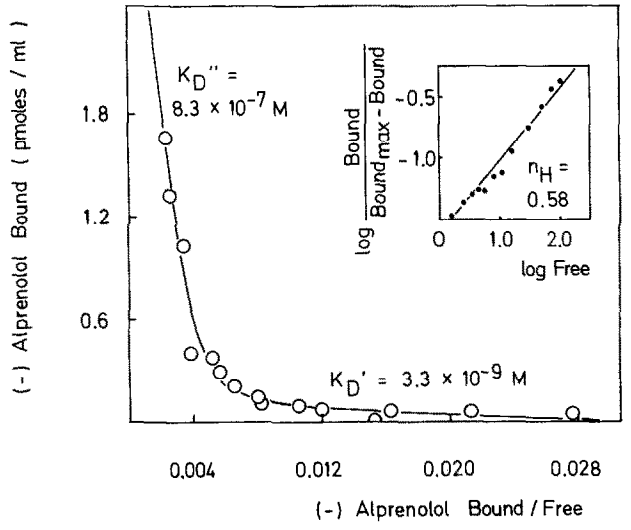


Fig. 4. Equilibrium binding of (−)-[^{3}H]dihydroalprenolol to lung tissue; methods as in Fig. 3. The data are indicative of negative cooperativity.

Table 1. Values of the dissociation constants (K_D' and K_D'') and binding capacities (R_1 and R_2) of cardiac and lung tissue for (−)-[^{3}H]dihydroalprenolol

	K_D' (M)	K_D''	R_1 (pmoles/mg protein)	R_2
Cardiac tissue	6.0×10^{-9}	4.3×10^{-6}	0.18	22.80
Lung tissue	3.3×10^{-9}	8.3×10^{-7}	0.13	5.86

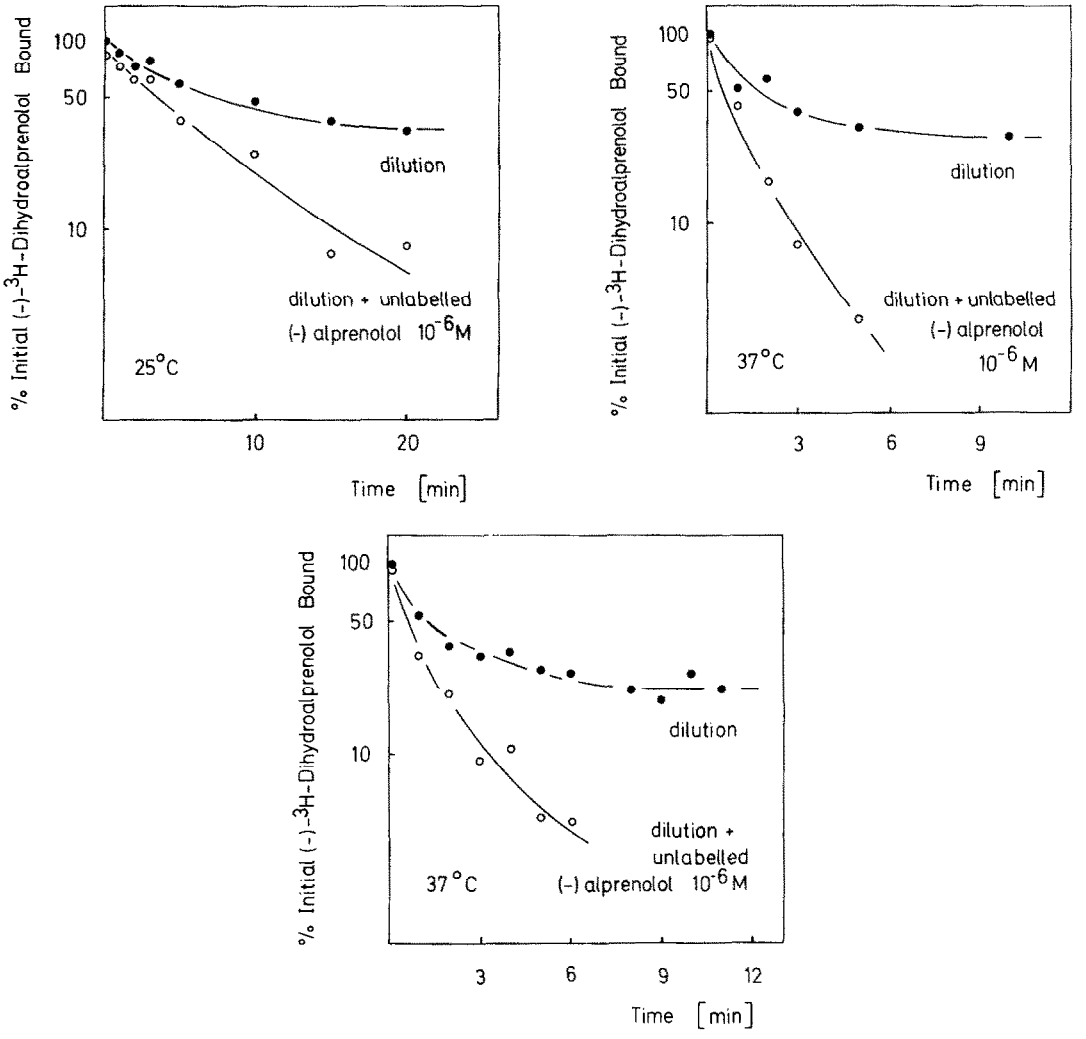


Fig. 5. Dissociation kinetics of (−)-[^{3}H]dihydroalprenolol in the absence (●—●) and presence (○—○) of 10^{-6} M unlabelled (−)alprenolol at different temperatures and tissues. (a) Membranes of cardiac tissue (8.2 mg protein/ml) were incubated with (−)-[^{3}H]dihydroalprenolol (6.4 nM) for 15 min at 25°. Aliquots of 100 µl each containing the usual assay (see Materials and Methods) were transferred to a series of tubes that contained 10 ml of 7.5 mM Tris–HCl, 2.5 mM $MgCl_2$, pH 7.4, with and without 10^{-6} M unlabelled (−)alprenolol at indicated intervals. The radioactivity bound to the membranes, expressed as percentage of the radioactivity at $t=0$, is plotted as a function of the time after the dilution of the membranes ($t=0$ refers to the time immediately after 1:100 dilution). Each point is the mean of duplicate determinations and specific binding (as defined in Materials and Methods) is subtracted. (b) Membranes of cardiac tissue were incubated for 15 min at 37°, further proceedings as in (a). (c) Membranes of lung tissue were incubated for 15 min at 37°, further proceedings as in (a).

DHA binding sites. The dissociation of labelled β-adrenergic antagonists from their binding sites is enhanced in the presence of 10^{-6} M unlabelled antagonists as compared with the dissociation rate by dilution of the incubation alone (Fig. 5a–c). The increased dissociation rate indicates that after occupation of empty binding sites (i.e. binding sites not filled by the low concentration of labelled β-blocking agent) the affinity of the sites already occupied by the radioligands is decreased. The affinity constant is lowered with increased occupancy of the receptor population. This is usually referred to as negative cooperativity [20]. The process is temperature dependent. (Fig. 5a and b). Similar results are obtained by measuring the dissociation kinetics of lung tissue in the same way. (Fig. 5c).

The following control experiments were performed to demonstrate that the effect is not an artefact (Fig. 6) [21]. To exclude the possibility of rebinding of labelled β-adrenergic antagonists during the dissociation phase the incubated membranes were divided. To one half of the membrane preparation was added an equal aliquot of membranes which had not been exposed to the radioligand before. To the second half of the preparation was added an equal volume of buffer. The two incubation media were then rapidly diluted in the presence and absence of 10^{-6} M unlabelled (−)alprenolol.

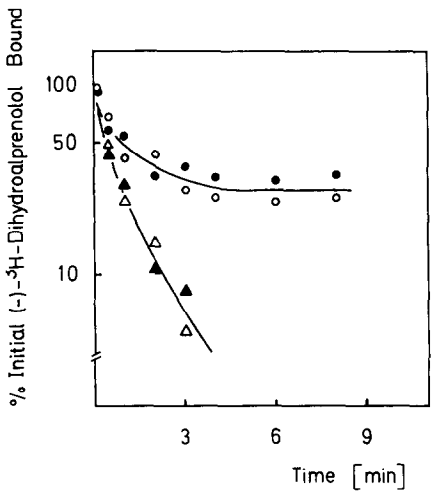


Fig. 6. Effect of addition of untreated membranes (i.e. membranes without bound (−)-[^{3}H]dihydroalprenolol) on the dissociation rate of (−)-[^{3}H]dihydroalprenolol. Membranes were incubated as described in Fig. 5. An equal aliquot of membranes (without bound (−)-[^{3}H]dihydroalprenolol) was then added to one half of the incubation volume; an aliquot of buffer was added to the other half. The two incubation media were then rapidly diluted in the presence and absence of 10^{-6} M unlabelled (−)alprenolol (in the presence of 10^{-6} M (−)alprenolol: (▲——▲) membrane plus an aliquot of membranes; (△——△) membranes plus an aliquot of buffer, in the absence of 10^{-6} M (−)alprenolol; (●——●) membrane plus an aliquot of membranes; (○——○) membrane plus an aliquot of buffer. This experiment demonstrates that no rebinding occurs. Each point is the mean of duplicate determinations and unspecific binding (as defined in Materials and Methods) is subtracted.

The rate of dissociation of (−)-[^{3}H]dihydroalprenolol was not affected by the addition of fresh membranes.

If rebinding had occurred, this should have been magnified by the greater than 2-fold increase in unoccupied receptor concentration achieved by addition of fresh membranes (Fig. 6), which should then have resulted in increased radioactivity on the membranes. In order to demonstrate that there are no effects involved due to unspecific binding sites the experiments for dissociation kinetics were performed in parallel with membranes from cardiac and lung tissue heated at 37° for 23 hr. According to previous results demonstrating the absence of specific binding sites in these heated membranes [10], there was no difference between the dilution effect alone and dilution with additional 10^{-6} M (−)alprenolol (Fig. 7).

DISCUSSION

Recently, hormone and drug binding sites have been characterized in membranes from different tissues and in several species. The characterization of specific β-adrenergic receptors through the use of labelled β-blocking agents such as (−)-[^{3}H]dihydroalprenolol, [^{125}I]hydroxybenzylpindolol or (±)-[^{3}H]propranolol hydrochloride has been introduced by Lefkowitz *et al.* [2], Aurbach *et al.* [1] and Atlas *et al.* [22]. Information on β-adrenergic receptors has been accumulating since then [23–26].

Lately, evidence has been obtained for negative cooperativity among binding sites located on the cell membranes [5], meaning that the affinity of the receptor for the drug or hormone decreases with increasing drug or hormone concentration. Limbird *et al.* [21], who showed this 'negative cooperativity' of β-adrenergic receptors in frog erythrocytes, proposed the following criteria for this phenomenon: (1) curvilinear Scatchard plot or slope of the Hill plot $n_H < 1$; (2) the presence of unlabelled hormones increases the dissociation rate; (3) the dissociation rate of the ligand–receptor complex is not affected by the addition of untreated membranes.

Thus, there are two questions; firstly, can negative cooperativity also be found in β-adrenergic receptors of cardiac and lung tissue of mammalians? and secondly, is the effect measured an expression of specific binding sites?

Negative cooperativity among binding sites has been suggested to exist for insulin receptors in rat liver membranes and cultured lymphocytes [6] and guinea pig liver membranes [27]. In turkey erythrocytes [28, 29] there is apparently no such phenomenon with β-adrenergic receptors. Negative cooperativity among STH-receptors does not seem to exist either [6]. These findings suggest that there are some drugs or hormones which induce negative cooperativity among their receptors in all tissues (insulin), some drugs or hormones which do not induce negative cooperative effects at all (STH) and some, which induce negative cooperativity only in certain species (β-adrenergic blockers in frog but not in turkey erythrocytes). Thus, negative cooperativity may reflect certain biological properties of mem-

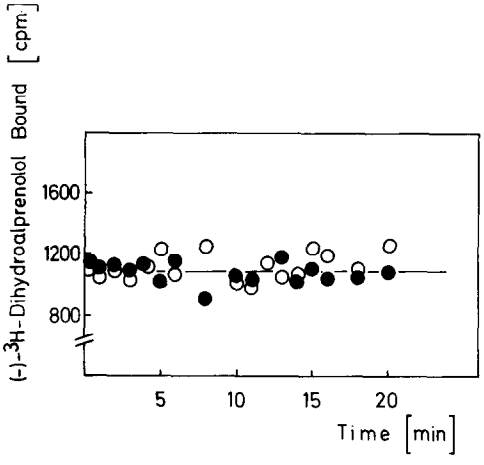


Fig. 7. Dissociation kinetics of (−)-[^{3}H]dihydroalprenolol in the absence (●——●) and presence (○——○) of 10^{-6} M unlabelled (−)alprenolol at unspecific binding sites. Membranes of cardiac tissue (8.0 mg protein/ml) were heated for 23 hr at 37°; afterwards the denatured membranes were incubated with (−)-[^{3}H]dihydroalprenolol (6.4 nM) for 15 min at 37°. Aliquots of 100 μl each containing the usual binding assay (see Materials and Methods) were transferred to a series of tubes that contained 10 ml of 7.5 mM Tris–HCl, 2.5 mM $MgCl_2$, pH 7.4, with and without 10^{-6} M unlabelled (−)alprenolol at indicated intervals. The radioactivity bound to the membranes, expressed in c.p.m., is not changed over the time with and without 10^{-6} M (−)alprenolol, and the values amounted to 50% of total binding. Means of duplicate experiments.

branes not present in all membranes. Our experiments performed with guinea pig cardiac and lung tissue fulfill the criteria of specific binding as defined by Lefkowitz [30] and Krawietz and Erdmann [10]. The mathematical calculations of our binding experiments (see Table 1 and Figs. 3 and 4) suggested two extreme conformations of one binding site with very different dissociation constants. All values between those two are, however, possible and real. The low affinity and high capacity receptor R_2 in our experiments can be explained as the one extreme of the conformationally changed R_1-receptors caused by negative cooperative effects. Nevertheless, our experiments demonstrate that binding of labelled alprenolol to the DHA binding sites makes further binding of unlabelled alprenolol more difficult, and the apparent affinity decreases steadily with increasing fractional occupancy. De Meyts and Roth [31] propose calculations of the resulting affinity and occupancy of binding sites in a special way. The 'unspecific binding' in our experiments can be measured exactly (identical to the binding capacity of membranes heated for 23 hr at 37° and not altered in different experimental conditions) [10]. No negative cooperative effect is seen at unspecific binding sites either by dilution or by diluting and additional 10^{-6} M (−)alprenolol (Fig. 7) [6]. The unspecific binding always determined in parallel with each membrane preparation was therefore subtracted from the total amount of (−)-[^{3}H]dihydroalprenolol binding.

In ouabain binding experiments with rat skeletal muscle cell membranes it has been shown that similar nonlinear Scatchard plots may result from a damaged membrane composition [32]. Other reasons for such curvilinear Scatchard plots have been discussed as for instance, a ligand–ligand interaction, by Cuatrecasas and Hollenberg [33]. Weiner and Jardetzky [34], however, demonstrated that epinephrine and norepinephrine did not polymerize at concentrations up to 0.1 M. It is therefore not to be expected that unlabelled alprenolol would interact with labelled alprenolol in the low concentrations (10^{-10} – 10^{-5} M) used in these experiments. Furthermore, a heterogenity of labelled and unlabelled ligand [35] has been refuted by chromatography. There was no difference between labelled and unlabelled alprenolol [36]. Thus, this pitfall may be excluded, too.

All of our experimental results speak very much in favour of the existence of negative cooperative effects in β-adrenergic receptors of guinea pig cardiac and lung tissue. Although the physiological role of negative cooperativity among receptor sites is still speculative [37], this phenomenon may provide for greater sensitivity to low concentrations of drugs or homones and may serve as a protective mechanism against acute and high elevations of these agents at the receptor sites. It might be that those hormones which are regularly secreted on acute demand within seconds and in excess (insuline, catecholamines) seem to induce negatively cooperative effects in their receptors, whereas other hormones (STH) act in a concentration-dependent way and do not show this peculiar phenomenon. Several possible mechanism have been proposed to explain these various cooperative effects. The most likely include conformational changes in the structure of oligomeric receptors, polymerization [38] or depolymerization of receptors in the fluid membrane [39] or a combination of these.

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