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Shallow Agonist Competition Binding Curves for β-Adrenergic Receptors: The Role of Tight Agonist Binding

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SUMMARY

The β_2-adrenergic receptors of bovine trapezius muscle membranes demonstrate tight agonist binding as a result of the formation of complexes between agonists, receptors, and N_s. Preincubation of the membranes with (−)-isoproterenol (followed by washing) causes a time- and concentration-dependent decrease in the number of radioligand-binding sites to a plateau value of 41.5 ± 4%. The affinity of the remaining sites for the radioligand (−)-[^{3}H]dihydroalprenolol is unchanged. This decrease is stable under radioligand binding conditions but is readily reversed in the presence of GTP. The isoproterenol/(−)-[^{3}H] dihydroalprenolol competition binding curves are shallow. Such a phenomenon is usually interpreted in terms of two interconvertible affinity states of the receptor: the high affinity state reflecting the coupling of the agonist·receptor complex to N_s and the low affinity state not interacting with N_s. However, the competition curves undergo time-dependent shifts to the left. This apparent non-equilibrium can be explained by a model in which tight agonist binding to part of the receptor population is included. The usual computerized interpretation of the competition binding curves do not allow the correct evaluation of agonist binding parameters in the presence of tight agonist binding.

It is now clear that the first steps in β-adrenergic signal transmission involve the binding of an agonist (H) to a β-adrenergic receptor (R) and the subsequent coupling of the agonist-receptor complex (H·R) to a stimulatory guanine nucleotide-regulatory protein (N_s) (Ref. 1 and references therein). The antagonist-bound receptors do not undergo such coupling to N_s. For the receptors in purified membrane preparations this functional difference is also reflected by the shallow agonist-radioligand competition binding curves as compared to the steep curves for antagonists (2, 3). Under equilibrium binding conditions, the shallow competition curves are compatible with an apparent heterogeneity of the receptors and have been interpreted in terms of two subpopulations (R_H and R_L) with high (K_H) and low affinity (K_L) for the agonist, respectively. The curves can be computer analyzed by linear transformation of the experimental data (Hofstee plot) (4) or by a nonlinear least squares curve-fitting procedure (3). Both methods are based on the law of mass action and provide estimates for the number as well as the respective dissociation constants (K_H and K_L) of the two receptor subpopulations. The sites demonstrating high affinity for the agonist have been proposed to reflect H·R·N_s complex formation and are detectable only in the absence of guanine nucleotides. The low affinity sites do not interact with N_s (5). This hypothesis was confirmed by reconstitution experiments involving the incorporation of pure receptors and pure N_s in liposomes (6).

A number of studies reported that the preincubation of membrane preparations from frog erythrocytes, rat heart, or rat lung with catecholamines causes a partial decrease in the number of receptors, i.e., antagonist-binding sites (7–9). This phenomenon, attributed to tight agonist binding, is distinct from the *in vivo* mechanism of desensitization and occurs only in broken cell preparations (10). Using the radiolabeled agonist [^{3}H]hydroxybenzyl isoproterenol, Williams *et al.* (11) were the first to demonstrate that agonists are able to undergo quasi-irreversible binding to β-adrenergic receptors in frog erythrocyte membranes. The requirement of H·R·N_s complex formation was shown by the stabilization of the binding of [^{3}H] hydroxybenzyl isoproterenol in the presence of Mg ions and by its rapid destabilization by GTP.

It is difficult to reconcile the quasi-irreversible nature of tight agonist binding with the usual interpretation of agonist competition curves. This interpretation is indeed based on the major assumption that receptor interaction with all ligands is

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ABBREVIATIONS: N_s, guanine nucleotide-binding stimulatory component of adenylate cyclase; [^{3}H]DHA, (−)-[^{3}H]dihydroalprenolol.

reversible. In the present study we demonstrate that β-adrenergic agonists can undergo tight binding to the β_2-adrenergic receptors in membranes of bovine trapezius muscle. Competition binding curves of (−)-isoproterenol/[^{3}H]DHA are shallow and show time-dependent shifts to the left. These data are incompatible with a situation of equilibrium binding. In contrast, the data fit with models in which a time-dependent decrease in receptor number by tight agonist binding is included. In conclusion, we suggest that the usual computerized interpretation of the competition binding curves (3–5) does not allow the correct evaluation of agonist binding parameters in the presence of tight agonist binding.

Materials and Methods

Chemicals. (−)-Isoproterenol bitartrate was from Sigma. (−)-[^{3}H]DHA (92 Ci/mmol) was obtained from New England Nuclear. All other chemicals were of analytical grade.

Preparation of membranes. Bovine musculus trapezius membranes were prepared as described by IJzerman *et al.* (12). Protein concentrations were determined by the method of Lowry *et al.* (13), using bovine serum albumin as standard.

Preincubation with isoproterenol. Membranes (4 mg/ml) were preincubated at 30° in Tris buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 140 mM NaCl) with (2.10^{-7} M) (−)-isoproterenol for increasing periods of time (0–60 min), or with increasing concentrations of (−)-isoproterenol (2.10^{-8}–1.10^{-6} M) for 30 min. The preincubation was terminated by the addition of 1 volume ice-cold Tris buffer and centrifugation for 2 min in an Eppendorf centrifuge (15,000 × *g*) at room temperature. The precipitated membranes were resuspended in 1 ml of fresh buffer and the procedure was repeated two times.

Binding of [^{3}H]DHA. Membrane suspensions (1–2 mg/ml) were incubated with the indicated concentrations of [^{3}H]DHA and (−)-isoproterenol for 10–60 min at 30° in Tris buffer. All assays were performed in duplicate in a final volume of 300 μl. The binding reaction was terminated by the addition of 4 ml of ice-cold Tris buffer and filtration through Whatman GF/B glass fiber filters. Each filter was washed three more times with an additional 4 ml of buffer. The radioactivity on the filters was assessed in a Packard liquid scintillation spectrometer. Specific binding was obtained by subtracting the nonspecific binding (i.e., binding in the presence of 20μM (−)-isoproterenol) from the total binding. In all figures and tables, bound radioligand refers to specific binding as defined above. Nonspecific binding was 10–15% of total binding.

Data analysis. The Scatchard plots (14) of the saturation binding curves were rectilinear, so that the total receptor number (B_{max}) and the equilibrium dissociation constant (K_D) for [^{3}H]DHA could be calculated by linear regression analysis. Competition binding curves were analyzed by two computer programs: one based on the linear transformation of the experimental data (Hofstee plots) (15) as described by Minneman *et al.* (4), and the other based on a nonlinear least squares curve-fitting procedure (12) virtually identical to the one described by De Lean *et al.* (5).

Results

Bovine trapezius muscle is a homogeneous source of β_2-adrenergic receptors (12). Binding of the radioligand [^{3}H]DHA reaches equilibrium within less than 10 min at 30° and remains stable for at least 60 min (Table 1). Scatchard plots (14) of [^{3}H]DHA binding for 10, 30, and 60 min are all linear and superimposable; the B_{max} and K_D values at the different incubation times are listed in Table 1.

Preincubation of bovine trapezius muscle membranes with 0.2 μM isoproterenol causes a time-dependent decrease in the number of [^{3}H]DHA-binding sites until a plateau value is reached after 30 min (Fig. 1). Preincubation of the membranes for the same time period with buffer only has no effect on [^{3}H]DHA binding (data not shown). The Scatchard plots of control and isoproterenol-incubated membranes (Fig. 2) demonstrate that the isoproterenol-mediated decrease in radioligand-binding sites is due to a diminution in B_{max} and not to a change in affinity for [^{3}H]DHA. Such an agonist-mediated decrease in

TABLE 1

Binding characteristics of ^{3}H-DHA

Bovine trapezius muscle membranes were incubated for the indicated periods of time with increasing concentrations of [^{3}H]DHA (0.2–4 nM) after which binding was measured. The binding parameters were calculated by Scatchard analysis (n = 3).

Incubation time	[^{3}H]DHA binding characteristics	
	B_{max}	K_D
min	*fmol/mg*	*nM*
10	80 ± 4	1.2 ± 0.2
30	79 ± 3	1.3 ± 0.3
60	82 ± 3	1.3 ± 0.2

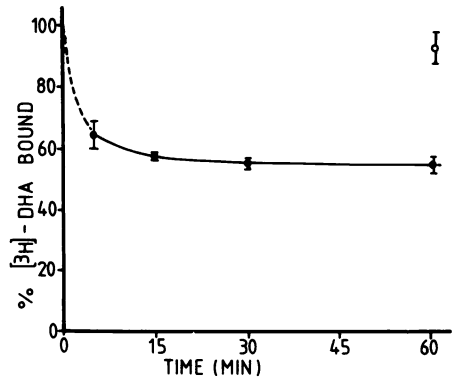

Fig. 1. Isoproterenol-mediated decrease in β-adrenergic receptor number in bovine trapezius muscle membranes as a function of the preincubation time. The membranes were preincubated with 0.2 μM isoproterenol (●) or with isoproterenol in the presence of 1 mM GTP (○), after which the membranes were washed and assayed for [^{3}H]DHA binding. Control binding, referring to membranes preincubated with buffer only, remained constant throughout the duration of the experiment.

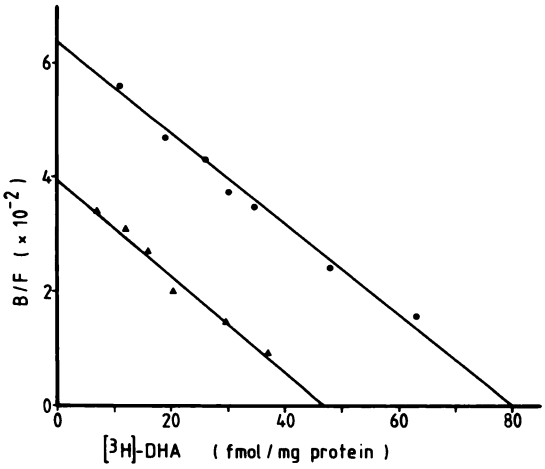

Fig. 2. Scatchard analysis of the specific binding of [^{3}H]DHA to native and isoproterenol-pretreated membranes. Membranes were preincubated for 30 min either with buffer alone (●) or with 0.2 μM isoproterenol (▲), washed, and subsequently incubated with increasing concentrations of [^{3}H]DHA (0.2–4 nM). The data shown are representative of those obtained in three similar experiments. ●, B_{max} = 80.4 fmol/mg of protein, K_D = 1.2 nM; ▲, B_{max} = 46.8 fmol/mg of protein, K_D = 1.1 nM.

radioligand binding has previously been attributed to the tight binding of agonists (7–9). In Figs. 1 and 2 it is also clearly shown that only part of the receptors undergo tight agonist binding. There is an average loss in receptor-ligand binding of 41.5% ± 3.8 (n = 8). The possibility of a time-dependent degradation of N_s as a possible cause for the partial nature of tight agonist binding has been ruled out. Indeed, preincubation of the membranes with buffer only at 30° for 10, 30, and 60 min does not interfere with subsequent tight agonist binding of isoproterenol. In the absence of guanine nucleotides, the tight binding of agonists is a very stable event. There is no change in binding when isoproterenol-pretreated membranes are incubated with [^{3}H]DHA for 10, 30, and 60 min at 30°. Hence, no significant dissociation of the agonist occurs. GTP is known to provoke the dissociation of the H·R·N_s complex. The addition of 1 mM GTP to the preincubation medium effectively prevents the agonist-mediated decrease in the number of [^{3}H]DHA-binding sites (Fig. 1).

The competition binding curves with the unlabeled antagonist propranolol follow the law of mass action and are superimposable for incubation times of 10 and 60 min (Fig. 3). In contrast, the isoproterenol/[^{3}H]DHA competition binding curves are shallow. Moreover, from Fig. 4 it appears that, as the time of incubation is prolonged (10–60 min), the curves are shifted to the left. Computer-assisted analysis of these shallow curves, following a two-site model, using programs derived from both Minneman *et al.* (4) and De Lean and co-workers (3, 5), yielded very similar interpretations; the proportion of high affinity sites as well as the affinity ratio between both receptor populations for isoproterenol (K_L/K_H) increase in a timewise fashion (Table 2). Under the usual incubation conditions (10–30 min at 30°), equilibrium is apparently not reached. Time-dependent shifts in the agonist competition binding curves appear to be associated with the ability of part of the receptors to undergo functional coupling to N_s. Indeed, the isoproterenol competition curves are superimposable for incubation times of

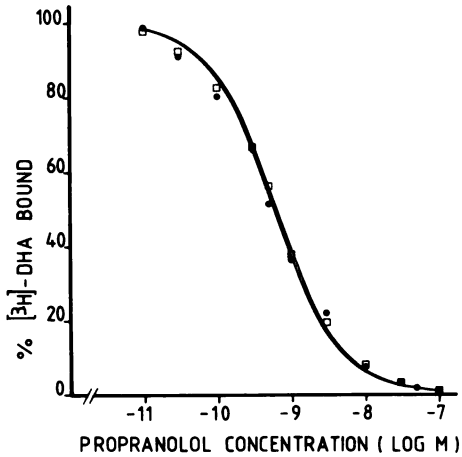

Fig. 3. Propranolol/[^{3}H]DHA competition binding as a function of the incubation time. Bovine trapezius muscle membranes were incubated with 1 nM [^{3}H]DHA in the presence of the indicated concentrations of the β-adrenergic antagonist propranolol for 10 (●) or 60 (□) min, after which binding was measured. Binding is expressed as percentage of control; i.e., binding in the presence of buffer only. Values are the mean of duplicate determinations in a single representative experiment. The competition binding data yielded straight Hofstee plots and, hence, followed the law of mass action with K_I values of 0.33 nM (●) and 0.30 nM (□). The curves are computer-drawn.

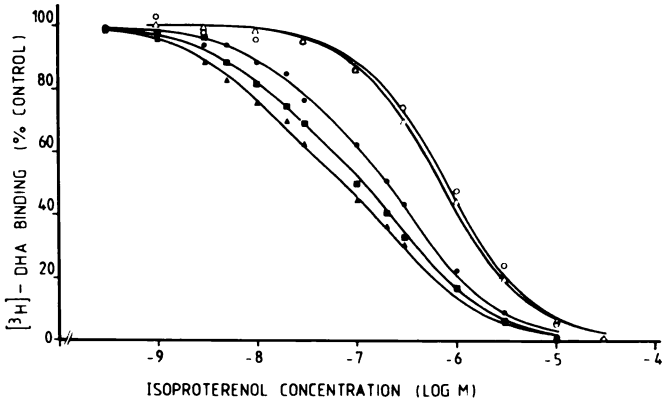

Fig. 4. Isoproterenol/[^{3}H]DHA competition binding as a function of the incubation time. The membranes were incubated with 1 nM [^{3}H]DHA and increasing concentrations of isoproterenol for 10 (●), 30 (■), and 60 (▲) min. Control binding (100%) was measured in the presence of buffer only. Values are the mean of duplicate determinations in three independent experiments. The competition binding data were analyzed by the computerized iterative method described by Minneman *et al.* (4) and by the nonlinear curve-fitting method described by De Lean *et al.* (5). The calculated parameters are listed in Table 2. The curves are computer-drawn using the data obtained by iteration. Isoproterenol competition binding was also performed in the presence of 1 mM GTP for 10 (○) and 60 (△) min. The competitive binding data followed the law of mass action with K_I values of 370 nM and 330 nM, respectively.

TABLE 2

Isoproterenol/[^{3}H]DHA competition binding as a function of the incubation time

The competition binding data, shown in Fig. 4, were analyzed by the computerized iterative method described by Minneman *et al.* (4) and by the nonlinear curve-fitting method described by De Lean *et al.* (5). In the former method the K_H and K_L values were calculated from their respective IC_{50} values according to the method of Cheng and Prusoff (16).

Incubation time	Calculated binding parameters for isoproterenol							
	Nonlinear curve fitting				Hofstee iteration			
	R_H	K_H	K_L	K_L/K_H	R_H	K_H	K_L	K_L/K_H
min	%	*nM*			%	*nM*		
10	32	12.9	228	17.6	30	11.6	201	17.3
30	45	8.8	188	21.4	38	6.6	172	26.0
60	46	5.2	170	33.0	46	5.3	158	29.7

10 and 60 min when GTP is included in the incubation medium (Fig. 4) or when using an agonist-pretreated membrane preparation in which the coupling-prone receptors are masked by tight agonist binding (Fig. 5). Under these two particular conditions, the competition curves become steep and display the same affinity for the agonist.

Discussion

In this investigation, we show that the preincubation of bovine trapezius muscle membranes with isoproterenol causes a time-dependent decrease in the β_2-adrenergic receptor number to a plateau value of approximately 60% of the initial amount (Fig. 1). This effect can be ascribed to the formation of a very stable agonist-receptor complex (i.e., tight agonist binding) and is dependent on coupling with N_s since it is effectively prevented by 1 mM GTP (Fig. 1).

One interesting point is that only a fraction of the receptors is involved in the tight binding of agonists in the membrane preparations investigated until now. Functional coupling of only part of the β_2-adrenergic receptors to N_s is also illustrated

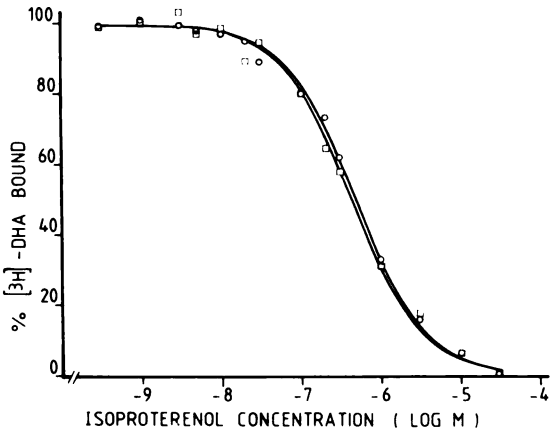

Fig. 5. Isoproterenol/[^{3}H]DHA competition curves on agonist-pretreated membranes. Membranes were preincubated with 0.2 μM isoproterenol during 30 min, resulting in a maximal decrease in the receptor number (Fig. 1). Competition binding occurred for 10 (○) and 60 (□) min as described in the legend of Fig. 4. The competition binding data followed the law of mass action with K_i values of 216 nM and 235 nM, respectively.

by the shallow agonist-radioligand competition binding curves. Several computer models have been developed for the analysis of these curves (4, 5); all assume that the curves are associated with agonist binding states of high and low affinity (i.e., R_H and R_L with the equilibrium dissociation constants K_H and K_L).

On the basis of the ternary complex model, De Lean and collaborators (5) attributed the low agonist affinity state to H·R and the high affinity state to H·R·N_s. This involves two equilibrium reactions (characterized by the equilibrium dissociation constants K_1 and K_2)—the formation of the agonist-receptor complex and its coupling to N_s:

$$H + R \overset{K_1}{\rightleftharpoons} H{\cdot}R + N_s \overset{K_2}{\rightleftharpoons} H{\cdot}R{\cdot}N_s$$

The agonist competition binding data were analyzed by the ternary complex model using the following assumptions. 1) All ligands bind to the receptors in a reversible way and equilibrium is attained when binding is measured. 2) Potentially, all of the receptors can participate in a ternary complex. 3) There are stoichiometrically comparable amounts of receptors and N_s.

Although this model has been found to provide a good explanation for the experimental competition binding data, assumption 1 is not compatible with the time-dependent leftward shifts of the curves obtained in the present study (Fig. 4), or with the quasi-irreversible nature of the tight agonist binding process. Moreover, recent studies suggest that there is no stoichiometric limitation of N_s (16, 17). Hence, the partial nature of the tight agonist binding indicates that only those receptors involved in this process are able to undergo functional coupling to N_s.

To account for these new experimental findings, we propose a new "tight agonist binding" model which can be schematized by the reactions:

$$H + R_n \overset{K_1}{\rightleftharpoons} H{\cdot}R_n \quad (1)$$

$$H + R_c \overset{K_1}{\rightleftharpoons} H{\cdot}R_c + N_s \overset{K_2}{\rightleftharpoons} H{\cdot}R_c{\cdot}N_s \quad (2)$$

with $R_{total} = R_c + R_n$

This model describes the binding of agonists according to the following assumptions. 1) The receptors are divided into two non-interconvertible subpopulations (R_c and R_n) on the basis of their ability to undergo functional coupling to N_s in the presence of agonists; R_c is coupling-prone and R_n is not (17, 18). 2) There is no stoichiometric limitation of N_s (17, 19). 3) Coupling to N_s is likely to occur according to a two-step mechanism (5, 20, 21): the H-R_c interaction is followed by coupling of the H·R_c complex to N_s. The first step, characterized by the equilibrium dissociation constant, K_1, is identical for R_c and R_n and is rapid and reversible. 4) Tight agonist binding involves only the R_c sites.

Assumption 3 can be verified experimentally: the agonist competition binding curve is steep when using membrane preparations in which the R_c sites are masked (Fig. 5). Moreover, these curves are superimposable for incubation times of 10 and 60 min, indicating that the H-R_n interaction is at equilibrium under the experimental conditions used in this study.

The simplest explanation accounting for the tight agonist binding is that the step characterized by K_2 is very slowly reversible. Indeed, experiments using the alkylating reagent *N*-ethylmaleimide revealed that both the receptor and N_s undergo major conformational changes upon formation of the ternary H·R·N_s complex. The receptor locks the agonist, whereas N_s exposes a previously masked —SH group. Alkylation of N_s by *N*-ethylmaleimide freezes the complex and the locked agonist prevents subsequent detection of the involved receptors by radioligand binding (22, 23). Since the agonist remains locked as long as the receptor is functionally coupled to N_s, tight agonist binding is likely to be the consequence of a stable receptor N_s binding. The ability of guanine nucleotides, known to cause receptor N_s uncoupling, to prevent and even to reverse tight agonist binding is in full agreement with this contention (9).

The shallow binding curves result from the different reactions occurring at the R_n and R_c sites. The binding of agonists to the R_n sites is a simple bimolecular reaction (Scheme 1) which is at equilibrium after 10 min incubation at 30°. The processes occurring at the R_c sites are more complex. Two possibilities can be distinguished as a function of the rate of dissociation of the agonist from R_c.

a) Absence of tight agonist binding. If there is no stoichimetric limitation of N_s and the H·R_c-N_s coupling step is fast and reversible, it is not possible to distinguish the coupled from the uncoupled R_c sites the basis of differences in agonist affinity. The R_c sites will behave as one single class of noncooperative sites toward the agonist with an effective "macroscopic" constant (K_c), defined by the following equation: $K_c = K_1 * K_2'/(1 + K_2')$, with $K_2' = K_2/N_s$. Hence, total agonist competition binding curves can be analyzed by the usual computerized methods to yield the exact proportion of R_c (R_H) and R_n (R_L), K_c (K_H) and K_1 (K_L). Whereas the K_L/K_H ratio can differ as a function of the agonist's intrinsic activity (3), the number of R_H sites must remain constant. Such situations have been reported to occur for D_2-dopamine receptors (24).

b) Presence of tight agonist binding. The agonist competition binding curves for R_c will undergo leftward shifts with increasing incubation times (Fig. 5). Indeed, Motulsky and Mahan (25) have previously demonstrated that there is more radioligand bound to the receptors at intermediate time points than at equilibrium, in the presence of a slowly dissociating competitor. This is in accordance with the decrease of the isoproterenol K_H values observed in our studies (Table 2).

Finally, we should stress that the calculated K_H values are not the actual equilibrium dissociation constants if there is tight agonist binding. This effect can easily be evidenced by

the apparent decrease in radioligand binding after preincubation with agonist. It is thus clear that, regardless of the statistical goodness of fit found by the usual computer analysis, care should be taken in attaching molecular significance to the constants derived from agonist competition binding curves.

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