The (−)[^{3}H]dihydroalprenolol binding to rat adipocyte membranes: an explanation of curvilinear Scatchard plots and implications for quantitation of β-adrenergic sites

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Abstract In rat adipocyte membranes, both β-adrenergic agonists and β-adrenergic antagonists competed with (−)[^{3}H]dihydroalprenolol for high affinity (K_D 2–4 nM) and low capacity binding sites. The antagonists but not the agonists competed with (−)[^{3}H]dihydroalprenolol for lower affinity and higher capacity sites. The present studies were performed in order to characterize the adipocyte β-adrenergic receptor and distinguish it from low affinity, higher capacity sites which were heat-labile and not stereoselective. When isoproterenol was used to define the nonspecific binding, saturation studies showed a single binding site with a capacity of ~100 fmol/mg membrane protein (corresponding to ~50,000 sites/adipocyte). Binding was saturated by 10 nM (−)[^{3}H]dihydroalprenolol. Approximate K_D's of 2–4 nM were observed. Kinetic analysis of (−)[^{3}H]dihydroalprenolol binding provided an independent measurement of K_D between 0.75 and 1.1 nM. This binding site had the characteristics of a β_1-adrenergic receptor with the potency of isoproterenol > norepinephrine ≧ epinephrine as competitors of binding. Furthermore, the K_D of inhibition of (−)[^{3}H]dihydroalprenolol binding correlated with the Ki of inhibition by antagonists or Ka of activation by agonists of glycerol release in isolated adipocytes ($r = 0.968$, $P < 0.001$). These results suggest that β-adrenergic agonists compete with (−)[^{3}H]dihydroalprenolol for the high affinity binding site which represents the physiological site. Furthermore, the use of antagonists (propranolol, alprenolol) to define specific β-binding includes nonspecific site(s) as well as the β-adrenergic site. Previous characterization and quantitation of β receptors in rat fat cell membranes may have been in error by incorporating both types of binding in their measurement.—**Dax, E. M., J. S. Partilla, and R. I. Gregerman.** The (−)[^{3}H]Dihydroalprenolol binding to rat adipocyte membranes: an explanation of curvilinear Scatchard plots and implications for quantitation of β-adrenergic sites. *J. Lipid Res.* 1982. **23:** 1001–1008.

Supplementary key words nonspecific binding • agonist-antagonist binding • biphasic Scatchard plots • stereospecificity

The (−)[^{3}H]dihydroalprenolol binding site in rat adipocyte membrane has been reported to differ from β-adrenergic binding sites in other mammalian tissues. The dissociation constant for (−)[^{3}H]dihydroalprenolol in adipocytes has been reported to be 12–20 nM (1–3). This affinity is 3 to 20 times lower than in other tissues such as rat brain (4), kidney (5), heart (6), liver (7), and human adipocyte (8).

The brown fat β-adrenergic receptor of rats was also reported to have low affinity (K_D ~ 95 nM) and high capacity (9). In contrast, a subsequent study of isolated brown adipocytes of hamsters reported a K_D of 2–5 nM (10). The major difference between these two reports was the use in the latter (10) of much lower concentrations of (−)[^{3}H]dihydroalprenolol and lower concentrations of antagonist to define the nonspecific binding.

The binding of (−)[^{3}H]dihydroalprenolol to rat adipocyte membranes in saturation studies has been reported to yield curvilinear Scatchard plots (11) with upward concavity (1, 3). Two studies have demonstrated that the curvilinearity of the Scatchard plots is not explained by negative cooperativity (12, 13), and suggested that the adipocyte membrane possesses two or more classes of receptor sites with discrete affinities. On the other hand, the existence of multiple classes of receptors has not been supported by studies of the lipolytic response to adrenergic agents (14).

In the course of our own work on aging and lipolysis in rat adipocytes, we were obliged to quantitate β-adrenergic receptors (15). We therefore investigated the binding characteristics of (−)[^{3}H]dihydroalprenolol to epididymal fat cell membranes. This report distinguishes between specific β-adrenergic binding sites and nonspecific binding sites for which certain β-adrenergic antagonists may compete with (−)[^{3}H]dihydroalprenolol. The inclusion of the nonstereoselective binding may

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distort the quantitation of β-adrenergic binding in rat adipocytes.

MATERIALS

The following compounds were provided as gifts: (−) and (+) propranolol and practolol (Ayerst), (+) isoproterenol (Sterling-Winthrop), and (−) and (+) alprenolol (Hässle). Other β-adrenergic agonists and antagonists were obtained from Sigma Chemical Company. All other reagents were of analytical grade. (−)-[^{3}H]dihydroalprenolol (~48 Ci/mmol) was purchased from New England Nuclear Company.

Collagenase (*Clostridium histolyticum* type I, Lot no. 40K108) was purchased from Worthington. Glycerokinase (*E. coli*) and α-glycerophosphate dehydrogenase were purchased from Sigma and Calbiochem, respectively.

METHODS

Preparation of isolated adipocytes and adipocyte membranes

Epididymal fat pads were removed from Sprague-Dawley or Wistar rats and immediately placed in isotonic saline at 37°C. Isolated adipocytes were prepared essentially according to the method of Rodbell (16). Five mg of collagenase/g wet weight of tissue in 3 ml/g wet weight of Krebs Ringer bicarbonate buffer with 4% bovine serum albumin (pH 7.4) was used as we have previously reported (15). Following collagenase digestion, the isolated adipocytes were seived through silk mesh and separated from the infranatant by flotation. The cells were washed three times in fresh Krebs-albumin buffer.

Adipocyte membranes were prepared by homogenizing isolated adipocytes in 0.25 M sucrose with 0.01 M Tris–HCl, 1 mM EDTA (pH 7.4) in a Dounce homogenizer. The homogenate was centrifuged at 15,000 *g* for 15 min. The pellet was resuspended in 0.05 M Tris–HCl buffer with 10 mM $MgCl_2$ at pH 7.4 (assay buffer), washed, and resuspended. The preparation was used directly in the binding assays at a concentration of 2–3.5 mg/ml.

(−)[^{3}H]dihydroalprenolol binding assays

All tubes were set up in duplicate or triplicate. Drugs, (−)[^{3}H]dihydroalprenolol, and tissue were solubilized or suspended in the Tris–HCl assay buffer. The total volume of the assay was 250 μl. The binding reaction was commenced by adding the tissue suspension. The incubation was carried out at 30°C for 10 min unless otherwise stated. Under these conditions no more than 11 to 14% of the (−)[^{3}H]dihydroalprenolol (1 to 100 nM) was bound to the assay tube. Bound (−)-[^{3}H]dihydroalprenolol was separated from "free" (−)[^{3}H]dihydroalprenolol by rapid vacuum filtration over GF/C filters (Whatman). The filter was then washed with 16 ml of ice-cold Tris–HCl assay buffer. The filtration procedure was accomplished within 10 sec. Radioactivity on the filters was determined in Aqueous Counting Scintillant (Amersham).

Saturation data were treated according to Rosenthal's (17) modification of Scatchard analysis (11). Competition studies were carried out incubating adipocyte membranes with a single concentration of (−)-[^{3}H]dihydroalprenolol and increasing concentrations of the competing drug. The equilibrium dissociation constants (K_D) for β-adrenergic agonists and antagonists with the binding site were estimated using the equation of Cheng and Prusoff (18)

$$K_D = EC_{50}/(1 + [L]/K_D^*),$$

where EC_{50} is the concentration of competitor inhibiting 50% of the binding, L is the concentration of (−)[^{3}H]dihydroalprenolol, and K_D^* is the dissociation constant for (−)[^{3}H]dihydroalprenolol independently determined from Rosenthal analyses (K_D^* = 3 nM).

Heat treatment of adipocyte membranes

Adipocyte membranes were heated to 50°C for 60 min in the presence of 10^{-4} M isoproterenol as described by Baker and Potter (19). This method was shown to protect β-adrenergic receptors in canine atrial membranes.

Glycerol release

Isolated adipocytes (~14,000 adipocytes/ml) were incubated at 37°C for 60 min in Krebs albumin buffer. Glycerol release was measured in 200 μl of the infranatant by the enzymatic method of Weiland (20). The activation constant (Ka) is the concentration of agonist for half maximum stimulation. The inhibition constants (Ki) were calculated from the equation of Cheng and Prusoff (18)

$$Ki = [IC_{50}]/(1+ [\text{isoproterenol}]/Ka \text{ for isoproterenol}),$$

where $[IC_{50}]$ is the concentration of antagonist inhibiting half the stimulation in the presence of 10^{-7} M isoproterenol, and Ka for isoproterenol is the concentration of isoproterenol resulting in half maximal stimulation.

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RESULTS

Saturation experiments using propranolol to define specific binding

Saturation studies in rat adipocyte membranes performed as previously published (1, 2) indicated that there were 400–600 fmol receptors/mg membrane protein (**Fig. 1**). The approximate K_D of binding was 12–20 nM and binding saturated at 80 nM (−)[^{3}H]dihydroalprenolol. Transformation of these data according to the method of Rosenthal (17) yielded curvilinear plots with upward concavity. Inclusion of several concentrations of (−)[^{3}H]dihydroalprenolol at or below 10 nM identified a shoulder in saturation plots at about 10 nM (−)[^{3}H]dihydroalprenolol (Fig. 1).

Negative co-operativity

Experiments to test if the curvilinear Rosenthal plots were due to negative cooperativity were carried out as reported by Malbon and Cabelli (12) at 2, 12, and 20 nM (−)[^{3}H]dihydroalprenolol. At 2 nM (−)[^{3}H]-dihydroalprenolol, 60–80% of the putative high affinity sites were occupied (Fig. 1). Dissociation rates were identical in the absence or presence of 10^{-5} M (−)isoproterenol or 5×10^{-6} M (−)propranolol.

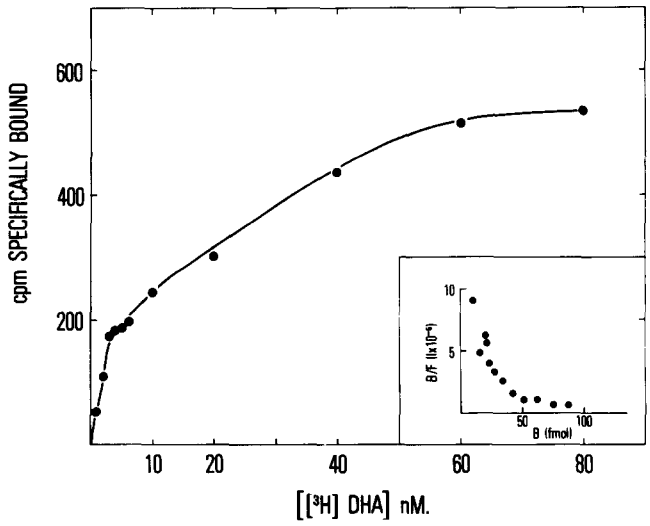

Fig. 1. Equilibrium binding study showing the saturability of 1 to 100 nM (−)[^{3}H]dihydroalprenolol in adipocyte membranes. Specific binding was defined as the difference between binding observed in the absence of (−) propranolol and binding observed in its presence (5×10^{-6} M). Membrane protein (300 μg) was incubated in a total volume of 250 μl 0.05 M Tris HCl buffer with 10 mM $MgCl_2$ (assay buffer) at pH 7.4 at 30°C for 10 min. Membrane-bound (−)[^{3}H]dihydroalprenolol was separated from unbound (−)-[^{3}H]dihydroalprenolol by vacuum filtration over glass fiber filters. The insert shows the Rosenthal analysis (17) of these data. The experiment is representative of at least six such experiments.

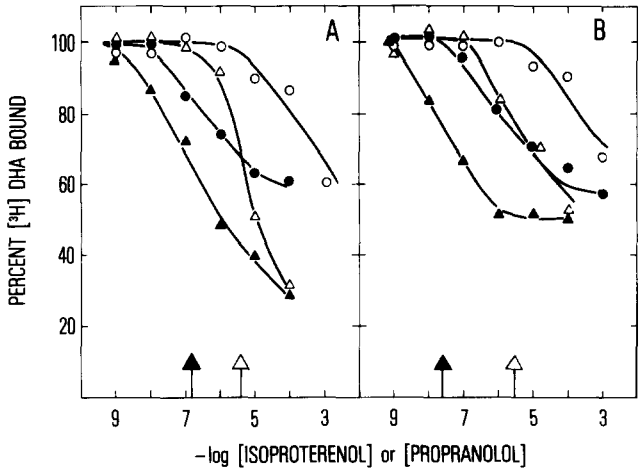

Fig. 2. Equilibrium binding studies showing the competition of binding of 6 nM (−)[^{3}H]dihydroalprenolol by the isomers of isoproterenol (●, ○) and propranolol (▲, △). Solid symbols show the (−) isomers. Panel A shows the competition in adipocyte membranes prepared as described in the methods section and washed three times in 100 volumes of assay buffer. Panel B shows competition studies in the same experiment except that the membranes were heat-treated (see Methods). The arrows indicate the concentration of (−) propranolol (⇑) and (+) propranolol (⇑) displacing 50% of the (−)[^{3}H]dihydroalprenolol from the membranes. Results were consistent in four different membrane preparations.

Differences between agonist and antagonist competition of (−)[^{3}H]dihydroalprenolol binding

Titration of the (−) and (+) isomers of the β-adrenergic agonists, isoproterenol, norepinephrine and epinephrine, and also the antagonists, alprenolol and propranolol, showed stereospecific competition of (−)[^{3}H]dihydroalprenolol binding. However, the antagonists competed more with (−)[^{3}H]dihydroalprenolol than the agonists at any given concentration of (−)[^{3}H]dihydroalprenolol. In the presence of maximally competing concentrations of isoproterenol, the antagonists were capable of further competition with (−)[^{3}H]dihydroalprenolol (**Fig. 2**). This further displacement of (−)[^{3}H]dihydroalprenolol has been directly related to the relative lipid-solubility of the competing ligand (21).

The difference between the K_D of the more potent (−) isomer and that of the (+) isomer of *isoproterenol* was 1000-fold at all concentrations of (−)[^{3}H]dihydroalprenolol tested, up to 20 nM. However, the difference in the K_D's between the stereoisomers of propranolol was less and decreased from 750-fold at 2 nM (−)[^{3}H]dihydroalprenolol to ~100-fold at 20 nM (−)[^{3}H]dihydroalprenolol (Fig. 2). Increasing concentrations of propranolol (up to 10^{-4} M) continued to compete with (−)[^{3}H]dihydroalprenolol in adipocyte membranes. In similar experiments where epinephrine and

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TABLE 1. [^{3}H]Dihydroalprenolol binding in adipocyte membranes

	10^{-5} M Isoproterenol	5×10^{-6} M Propranolol
B_{max} (fmol/mg)	96 ± 21	557 ± 67
K_D (nM)	1.9 ± 0.4	10.64 ± 0.73
n_H	1.1 ± 0.12	0.72 ± 0.03
Number of experiments	5	4

Comparison of results of saturation analysis of (−)[^{3}H]dihydroalprenolol from 1 to 100 nM using 10^{-5} M isoproterenol or 5×10^{-6} M propranolol to inhibit "specific binding" to obtain a measure of nonspecifically bound (−)[^{3}H]dihydroalprenolol. Data were analyzed according to the method of Rosenthal (17) to achieve capacity (B_{max}) and (−)[^{3}H]dihydroalprenolol dissociation constants (K_D). Hill coefficients (n_H) were achieved from the slope of linear regression analysis of plotting log ($B/[B_{max} - B]$) vs. "free" (−)[^{3}H]-dihydroalprenolol, where B was the (−)[^{3}H]dihydroalprenolol bound for a given concentration of (−)[^{3}H]dihydroalprenolol.

norepinephrine competed with (−)[^{3}H]dihydroalprenolol binding in fat cell membranes, there remained a component of binding that was inhibited by propranolol and alprenolol, but not by the agonists. At 6 nM (−)[^{3}H]dihydroalprenolol, the difference between the K_D's of the isomers of propranolol was 150-fold (Fig. 2).

In membrane preparations heat treatment (see Methods) altered the competition characteristics of propranolol but not of isoproterenol (Fig. 2). The difference in IC_{50} of competition between the isomers of propranolol was then increased to 750-fold. The IC_{50} for (−)propranolol increased from 1.5×10^{-7} M before heating to 2×10^{-8} M, suggesting the elimination of a nonstereospecific factor.

The binding capacities and K_D were compared in equilibrium binding saturation experiments using either 10^{-5} M isoproterenol or 5×10^{-6} M propranolol to suppress the "specific" binding. Concentrations of 1 to 100 nM (−)[^{3}H]dihydroalprenolol were used. The results are summarized in **Table 1.**

Differences between agonist and antagonist competition of (−)[^{3}H]dihydroalprenolol binding were also examined in saturation studies. "Specific" binding was defined either by 10^{-4} M propranolol in order to exaggerate the low affinity binding or by 10^{-4} M isoproterenol. Rosenthal transformation of these detailed saturation studies showed a curvilinear plot where propranolol was used but a linear plot where isoproterenol was used (**Fig. 3,** panel A). After heat treatment (see Methods) saturation studies with (−)[^{3}H]dihydroalprenolol (1–80 nM) indicated that the component of binding displaced by propranolol alone and resulting in the curvilinear Rosenthal plots was heat-labile and was not protected from heating by (−)isoproterenol (Fig. 3, panel B). The characteristics of the binding site protected by isoproterenol again were unchanged by heating.

Characterization of the adipocyte β-adrenergic receptor using isoproterenol to define specific binding

"Specific" binding was therefore redefined as the difference observed between (−)[^{3}H]dihydroalprenolol binding in the absence (total binding) and presence of 10^{-5} M isoproterenol (nonspecific binding). Under these conditions saturation studies showed that the binding sites saturated at 10 nM (−)[^{3}H]dihydroalprenolol with an approximate K_D of 2–4 nM (**Fig. 4**). Rosenthal transformation of these data yielded linear plots with K_D of 2–4 nM and a capacity of ~100 fmol/mg. This corresponded to about 50,000 receptors per adipocyte if it were assumed that one molecule of (−)[^{3}H]-dihydroalprenolol binds with one receptor. Hill plots gave slopes that did not differ significantly from unity (Table 1).

Kinetic studies

Specific binding of 2 nM (−)[^{3}H]dihydroalprenolol to adipocyte membranes was rapid. Equilibrium was

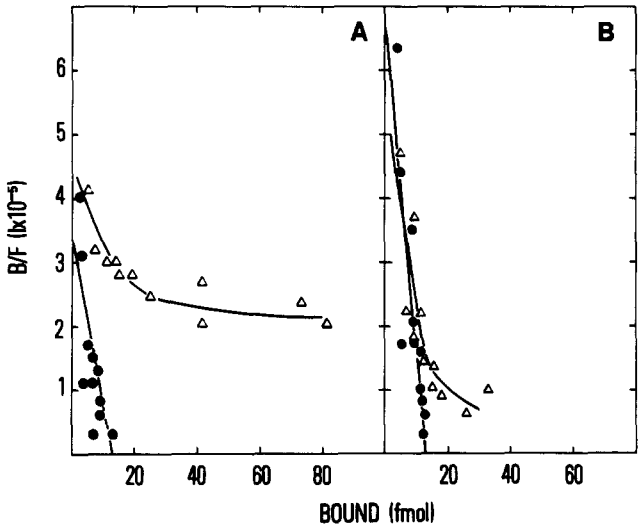

Fig. 3. Rosenthal analysis of equilibrium binding saturation studies using concentrations of (−)[^{3}H]dihydroalprenolol from 1 to 40 nM. Specific binding was defined either as the difference between binding in the absence and presence of 10^{-4} M (−) isoproterenol (●) or the difference between binding in the absence and presence of 10^{-4} M (−) propranolol (△). Incubation volumes and conditions are as described for Fig. 1. In panel A adipocyte membranes were prepared as described for Fig. 2, panel A; and in panel B, membrane preparations were heat treated as in Fig. 2, panel B. In the experiment shown, the binding capacity defined by isoproterenol was 114 and 128 fmol/mg in panels A and B, respectively. The dissociation constants were 3.8 and 1.9 nM. The capacities for [^{3}H]dihydroalprenolol binding competed by 10^{-4} M propranolol decreased from 1834 to 326 fmol/mg after heat treatment. The K_D decreased from 62.5 to 10 nM. The experiment was repeated with similar results.

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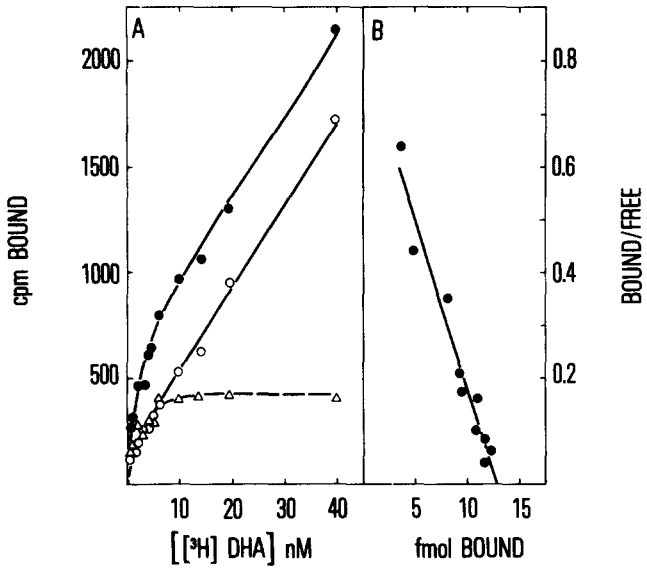

Fig. 4. Equilibrium binding studies using 1–40 nM (−)-[^{3}H]dihydroalprenolol. Specific binding (Δ – – – Δ) was defined as the difference between binding in the absence (●) and presence of 10^{-5} M isoproterenol (○). Adipocyte membrane preparation and incubation conditions were as described in Fig. 1. Rosenthal analysis of these data are shown in panel B. The results are consistent in at least ten experiments.

achieved by 5 min at 30°C (**Fig. 5**). Kinetic data (as illustrated) were used to calculate the rate constant, k_1, for the reaction $R_F + L \rightarrow RL$ where L represents (−)[^{3}H]dihydroalprenolol, R_F represents the free β-adrenergic receptor, and RL is the receptor: (−)-[^{3}H]dihydroalprenolol complex (2). The slope (k_{ob}) of the line in Fig. 5 (0.59 min^{-1}) provides an estimate of the observed forward rate constant for the pseudo first order reversible reaction in which the concentration of receptor (about 40 pM) is much less than the concentration of (−)[^{3}H]dihydroalprenolol (2 nM). The second order rate constant, k_1, is computed from $k_1 = (k_{ob} - k_2)/[L] = 1.57 \times 10^8$ M^{-1} min^{-1}, where [L] is the concentration of (−)[^{3}H]dihydroalprenolol and k_2 is the independently determined rate constant for the reaction $RL \rightarrow R + L$. Dissociation at 30°C followed first order kinetics with a rate constant, k_2, of 1.75×10^{-1} min^{-1} (**Fig. 6**). At 30°C, 80% of the specifically bound counts were dissociated at 10 min in the presence of excess isoproterenol or propranolol.

The ratio, k_2/k_1 of the rate constants gave an estimated equilibrium dissociation constant, K_D of 0.75 to 1.11 nM. These were in good agreement with the K_D derived from saturation studies.

Pharmacological specificity versus biological response

In isolated adipocytes the inhibition of isoproterenol (10^{-7} M)-stimulated lipolysis by antagonists or stimulation of lipolysis by agonists was related to the inhibition of (−)[^{3}H]dihydroalprenolol binding in adipocyte membranes by the same ligands. (Refer to Methods section.) In each case, the majority of binding sites showed typical β_1-adrenergic specificity. Isoproterenol had greater potency than norepinephrine or epinephrine (**Fig. 7** where K_D isoproperenol > norepinephrine ≧ epinephrine) in either stimulating lipolysis or competing with (−)[^{3}H]dihydroalprenolol for the binding site in the presence of 10^{-4} M GTP. The relationship between the K_D for the inhibition of binding according to the equation of Cheng and Prusoff (18) and the Ki or Ka of antagonist inhibition or agonist activation of lipolysis showed good correlation ($r = 0.948$, $P < 0.001$) with a slope of 0.925 which did not differ significantly from unity (Fig. 7).

Schild analysis (22) using 6×10^{-9} to 10^{-5} M (−) alprenolol to compete with the isoproterenol (10^{-8} to 10^{-4} M)-stimulated lipolytic response, showed that inhibition was competitive as the slope in three experiments approximated unity (data not shown). Since al-

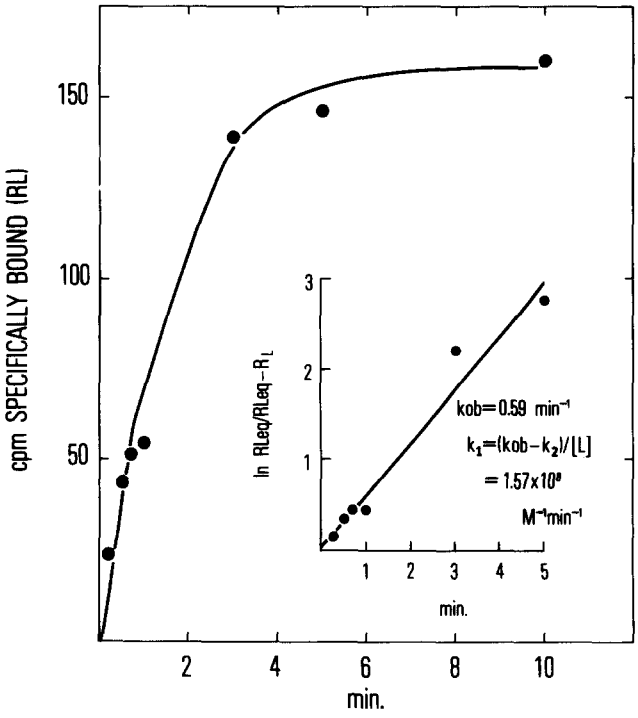

Fig. 5. Kinetics of specific (−)[^{3}H]dihydroalprenolol binding (RL) to adipocyte membranes at 30°C. Incubation conditions were as described in Fig. 1 except that the incubation times were varied appropriately. Nonspecific binding was defined as the binding in the presence of 10^{-5} M isoproterenol. The insert shows the pseudo first order rate plot of (−)[^{3}H]dihydroalprenolol binding of the same data. The slope of the line (k_{ob}) was determined by linear regression analysis ($r = 0.98$, $P < 0.001$). k_2 was obtained from the data in Fig. 6. The experiment was repeated four times with identical results.

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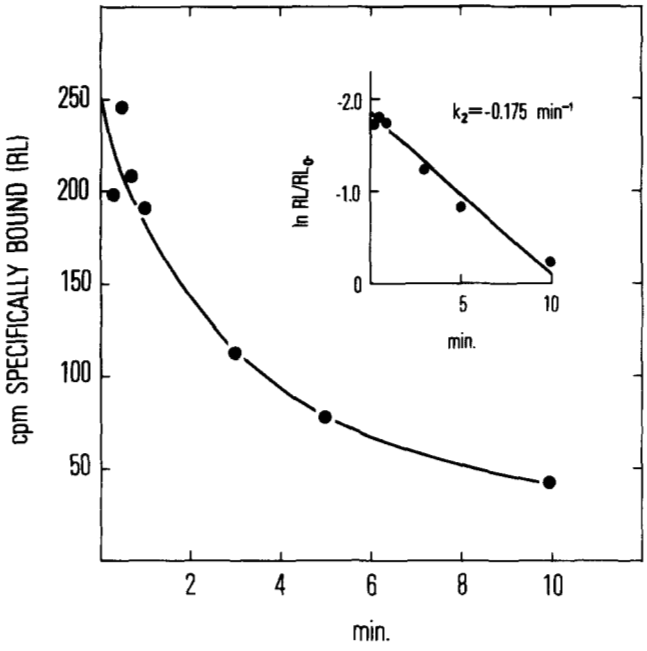

Fig. 6. Dissociation of (−)[^{3}H]dihydroalprenolol from adipocyte membranes at 30°C. Membranes were equilibrated with 2 nM (−)[^{3}H]dihydroalprenolol for 10 min at 30°C. Binding was reversed at time 0 min by addition of 10^{-5} M isoproterenol and the incubation mixture was poured over glass fibre filters and washed at the indicated times. The slope of the line provided an estimate of the dissociation constant (k_2) and was determined by linear regression analysis ($r = 0.98$, $P < 0.001$). The experiment was repeated three times with identical results.

prenolol showed extensive nonspecific binding in membrane preparations, 10^{-5} M phentolamine was included in the Krebs-albumin buffer. The estimated K_D in three experiments was 6.9 ± 1.7 nM.

DISCUSSION

Specific (−)[^{3}H]dihydroalprenolol binding is usually defined as the difference between binding in the absence (total binding) and presence of propranolol or alprenolol (nonspecific binding) at a concentration of 100 times the ligand's K_D. Under these conditions we observed data similar to those obtained earlier by others (1–3). However, when binding was examined in greater detail by using several concentrations of (−)[^{3}H]dihydroalprenolol less than 10 nM, the saturation plots showed a distinct shoulder at about 10 nM (−)[^{3}H]dihydroalprenolol. Rosenthal analysis (17) of these data yielded curvilinear plots.

Both saturation and competition experiments performed on heat-treated membranes along with those using isoproterenol to define the "nonspecific binding", showed that propranolol competed with (−)[^{3}H]-dihydroalprenolol for a low affinity, nonstereoselective, heat-labile site. We concluded curvilinearity of Rosenthal plots of adipocyte membranes is an artefact of using the antagonist propranolol to determine specific binding. The observation that propranolol may suppress a nonreceptor component of (−)[^{3}H]dihydroalprenolol binding is not a novel one. We have previously observed this in liver tissue of rats (23). Others have made similar observations in rat renal tissue (5), rat lung (24), guinea pig brain (25), and rat brain stem (26). Furthermore, in adipocyte membranes, it has clearly been shown that the more lipid-soluble ligands such as propranolol and alprenolol nonspecifically compete with (−)[^{3}H]-dihydroalprenolol for the membranes (21).

Identification of the heat-labile, nonstereospecific binding of (−)[^{3}H]dihydroalprenolol leads to redefinition of the quantitative aspects of (−)[^{3}H]dihydroalprenolol binding in rat adipocyte membranes. When the β-adrenergic agonist isoproterenol is used to define specific binding, quite different values for (−)[^{3}H]dihydroalprenolol binding are observed than when propranolol is the competing agent. With isoproterenol, even at concentrations up to 10^{-4} M, binding saturates at 10 nM (−)[^{3}H]dihydroalprenolol with K_D values of 2–4 nM. Rosenthal plots of these data are linear and give maximum binding capacity of 100 fmol/

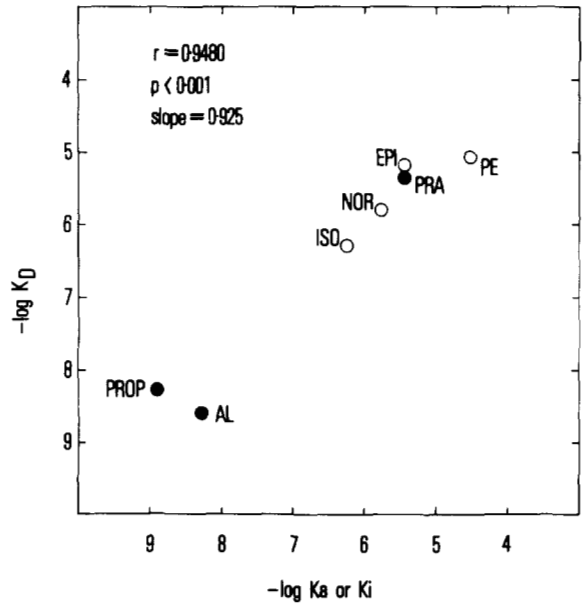

Fig. 7. Correlation between the stimulation of glycerol release by β-adrenergic agonists (○) and inhibition of glycerol release stimulated by 10^{-7} M isoproterenol by antagonists (●) in isolated adipocytes with (−)[^{3}H]dihydroalprenolol competition in the presence of 10^{-4} M GTP from membranes prepared from the same adipocytes. The logarithm of the K_a or K_i of glycerol release was determined by the method of Cheng and Prusoff (18). The slope of the line was determined by linear regression analysis and did not differ significantly from unity. PROP, (−)propranolol; AL, (−)alprenolol; ISO, (−)isoproterenol; NOR, norepinephrine; EPI, epinephrine; PRA, practolol; PE, phenylephrine.

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mg (~50,000 receptors/cell). Hill coefficients of these saturation data approach unity. These data indicate the presence of a single, high affinity (−)[^{3}H]dihydroalprenolol binding site on adipocyte membranes.

The inhibition of binding of (−)[^{3}H]dihydroalprenolol to fat cell membranes by β-adrenergic agonists and antagonists was correlated with the effect of lipolysis of the same β-adrenergic agents (Fig. 7). The pharmacologic potencies in inhibiting or stimulating glycerol release correlate with the potencies of these agents to inhibit (−)[^{3}H]dihydroalprenolol binding. These results support the contention that the binding site that we are defining is the physiologically relevant β-adrenergic receptor.

Several workers have described quantitative changes in adipocyte β-adrenergic receptor number under different physiological circumstances using concentrations of propranolol up to 10^{-5} M to define "specific" binding (1, 3, 27). Our results suggest that not only are the quantitative aspects of earlier reports in error as regards receptor number and affinity, but that these erroneous values may themselves be altered during physiologic manipulations. Thus, observed differences between groups of animals may be subject to even more than a systematic error. That such concerns have some basis in experimental fact is already evident. In liver tissue we have observed that a nonstereospecific binding which is competed for by propranolol is more obvious in liver membranes derived from adult rats (greater than 2 months or 150 g) as contrasted to younger rats (23). Similarly, we have observed that fat cell membranes from older rats (6–12 months) than used here tended to show a larger component of antagonist-displaceable heat-labile binding (data not shown). Others have recently discussed this possibility with quantitation of β-adrenergic receptors in aging human lymphocytes (28). The use of binding assays that employ concentrations of (−)[^{3}H]dihydroalprenolol greater than 10 nM and propranolol or alprenolol competition to define specific binding favors the measurement of displaceable nonstereospecific binding.

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