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Coexistence of *Beta*$_1$ and *Beta*$_2$ Adrenoceptors in Mammalian Lung: Evidence from Direct Binding Studies

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SUMMARY

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[^{3}H]Dihydroalprenolol ([^{3}H]DHA) binds to rat and rabbit lung membranes with a dissociation equilibrium constant (K_d) of about 0.5 nM and displays binding characteristics indicative of an interaction with *beta* adrenoceptors. The density of *beta* adrenoceptor binding sites was greater in rat than rabbit lung membranes, though the binding sites from both species had very similar affinities for nonselective agents such as (−)isoproterenol, (−)epinephrine, and (−)timolol, and exerted a similar degree of stereoselectivity between the isomers of propranolol. On the other hand, the affinity of *beta*$_1$ selective agents such as (±)practolol, (±)atenolol, and (−)norepinephrine was greater in rabbit lung membranes whereas the *beta*$_2$ agonist (−)erythro procaterol (OPC-2009) was considerably more potent on membranes prepared from rat lung.

However, detailed analysis of the competition curves displayed by the *beta*$_1$ and *beta*$_2$ selective drugs strongly suggest that *beta*$_1$ and *beta*$_2$ adrenoceptor binding sites coexist in different proportions within lung tissue in the two species (rat 25%, *beta*$_1$; 75% *beta*$_2$; rabbit 60%, *beta*$_1$; 40% *beta*$_2$). The significance of these findings to the effects of catecholamines on pulmonary function is discussed.

INTRODUCTION

The original observations and suggestion of Lands *et al.* (1) that *beta* adrenoceptors are not homogeneous has received substantial support (2, 3) though an organ-specific strict subdivision of *beta*$_1$ and *beta*$_2$ receptors has been the subject of some debate. For example, some workers have reported that adrenergic agonists and antagonists have a spectrum of affinities depending upon the tissue and species examined (4, 5) and have proposed that several *beta* adrenoceptor subtypes exist. On the other hand, it has been suggested that *beta*$_1$ and *beta*$_2$ adrenoceptors may be present in varying proportions in different tissues (6-8).

In an attempt to provide more direct evidence for the subclassification of *beta* adrenoceptors, we have recently examined the pharmacological characteristics of the binding sites specifically labeled in rat lung, heart, and brain membranes by the *beta* adrenergic ligand [^{3}H](−)-dihydroalprenolol, ([^{3}H]DHA). Our preliminary results suggested that in lung and cerebral cortex, at least, two distinct *beta* adrenoceptor binding sites co-exist (possessing properties described in intact preparations as *beta*$_1$ and *beta*$_2$) but that the proportions of the subtypes differ between the tissues (9, 10).[1] In the present investigation we have extended our studies on rat lung and made comparisons with the *beta* adrenoceptor of

[1] S. R. Nahorski, submitted for publication.

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rabbit lung since recent observations have indicated that rabbit airway smooth muscle is relatively insensitive to *beta* adrenergic agonists (11). In addition, the *beta* adrenoceptor coupled to adenylate cyclase in rabbit lung homogenates has been reported to possess surprising characteristics identical to those found in cardiac tissue ($beta_1$) (12). We have used direct ligand binding techniques to investigate the hypothesis that these differences may reflect coexistence in varying proportions in lung tissue of the two basic *beta* adrenoceptor subtypes.

MATERIALS AND METHODS

Materials. (−)[^{3}H]Dihydroalprenolol (48 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, England. (−)Isoproterenol HCl, (−)epinephrine HCl and (−)norepinephrine HCl were obtained from Sigma Chemical Company. The following drugs were kindly donated by the indicated companies. (+) and (−)Propranolol, (±)practolol and (±)atenolol (ICI Pharmaceuticals); (+) and (−)alprenolol, (±)H35/25 (AB Hässle); (±)oxprenolol and (±)para-oxprenolol (Ciba-Geigy); (−)timolol (Leo Laboratories); (±)salbutamol (Allen & Hanburys); (−)erythro procaterol (Otsuka).

Membrane preparation. Lungs were removed from male Wistar rats (100–150 g) or New Zealand White rabbits (1–3 kg) and dissected free of major bronchi. The tissue was homogenized in 10 vol. of buffer containing 5 mM Tris-HCl, 0.25 M sucrose and 1 mM $MgCl_2$, pH 7.4, using an Ultra-Turrax homogenizer (2 × 10 sec bursts) followed by 4 × 4 strokes of a motor-driven glass-Teflon homogenizer. The homogenate was passed through a single layer of cheesecloth and centrifuged at 480 *g* for 10 min at 4° to remove unbroken cells and connective tissue, which considerably hindered the subsequent rapid filtration in the binding assay. The supernatant was recentrifuged at 30,000 × *g* for 10 min. The final pellet was washed three times before resuspension in assay buffer (Tris-HCl 50 mM, pH 7.4).

Membranes could be stored at −40° without significant loss of binding activity.

Binding assay. Lung membranes (100–200 μg protein/100 μl) were incubated with (−)[^{3}H]DHA, 10,000–20,000 cpm (1–2 nM), except where indicated, in a final volume of 250 μl of assay buffer. All incubations were carried out at room temperature with shaking for 30 min. In competition experiments the displacing compound was added directly to the incubation mixture in a volume of 50 μl in replacement for 50 μl of assay buffer. Final concentrations were calculated accordingly. Incubations were terminated by the addition of 1 ml of ice-cold assay buffer followed by rapid vacuum filtration over Whatman GF/B glass fiber filters. Filters were then rapidly washed with 3 × 5 ml of assay buffer at room temperature. The radioactivity bound to the membranes trapped by the filters was measured in a Triton/Toluene scintillation mixture by counting in a Packard Liquid Scintillation counter at an efficiency of 40%.

Separate incubations were carried out in each assay in the presence of a high concentration of isoproterenol (200 μM) to assess nonspecific binding to the membranes. Specific binding was defined as total radioactivity bound minus nonspecific binding and was generally 85–90% of total counts bound. Binding of [^{3}H]DHA in all results and figures in this work refers to *specific binding* only.

Calculation of results. Data from some competition experiments have been graphically analyzed with a modified Scatchard analysis. In using this method certain assumptions have been made. The percent inhibition of [^{3}H]DHA binding has been equated with the absolute amount of competing drug bound to the receptors (i. e., the fractional occupancy of the binding sites by the competing ligand). This approximation is acceptable under the circumstances of these experiments, since although the competing ligand may have different affinity for the receptor subtypes, the radioactive ligand ([^{3}H]DHA) being displaced binds with equal affinity to both subtypes (the K_d of [^{3}H]DHA is identical in tissues containing either $beta_1$ or $beta_2$ subtypes predominantly). Secondly, in competition experiments the fraction of total [^{3}H]DHA bound to the membranes in the absence of competing ligand is less than

10% (see legend to Table 1). This would indicate that the "free" component of radioactive or competing ligand is relatively constant and that variations in this factor would be unlikely to influence the graphical analysis. It should be stressed that this type of analysis is only valid under the conditions described and that an accurate assessment of the affinities and proportion of receptor subtypes can only be achieved with the use of highly (>30 fold) selective agents.

RESULTS

The binding of [^{3}H]DHA to lung membranes of both species was saturable (Fig. 1a). The maximum number of binding sites at saturation, as assessed by Scatchard analysis (Fig. 1b), for rat lung was approximately twice that in rabbit lung. The equilibrium dissociation constant (K_d) for the reaction, defined as the concentration of [^{3}H]DHA at which half maximal binding occurred, was about 0.5 nM for both species.

The binding of [^{3}H]DHA to all lung membranes was rapid (t½ association, 4–5 min) and reversible (t½ dissociation, 10–15 min) at room temperature and the binding sites exhibited no cooperative interactions as assessed by Hill plots of the saturation curves (Hill coefficient, 1.1 and 1.0, for rat and rabbit, respectively). In view of the fairly long dissociation t½ at room temperature, the method of rapid filtration was satisfactory for separation of bound ligand. Binding of [^{3}H]DHA to the filters was insignificant (<0.1% of total added counts).

The binding was clearly stereoselective (Fig. 2) as (−)propranolol was approximately 100 times more potent than (+)propranolol in displacing [^{3}H]DHA from the membrane binding sites in both animals. In addition, in displacement experiments *beta* adrenergic agonists and antagonists (Fig. 3 and Table 1) exhibited relatively high affinity for the binding sites. Thus, in both animals specific [^{3}H]DHA binding was of high affinity, reversible, stereospecific, and ex-

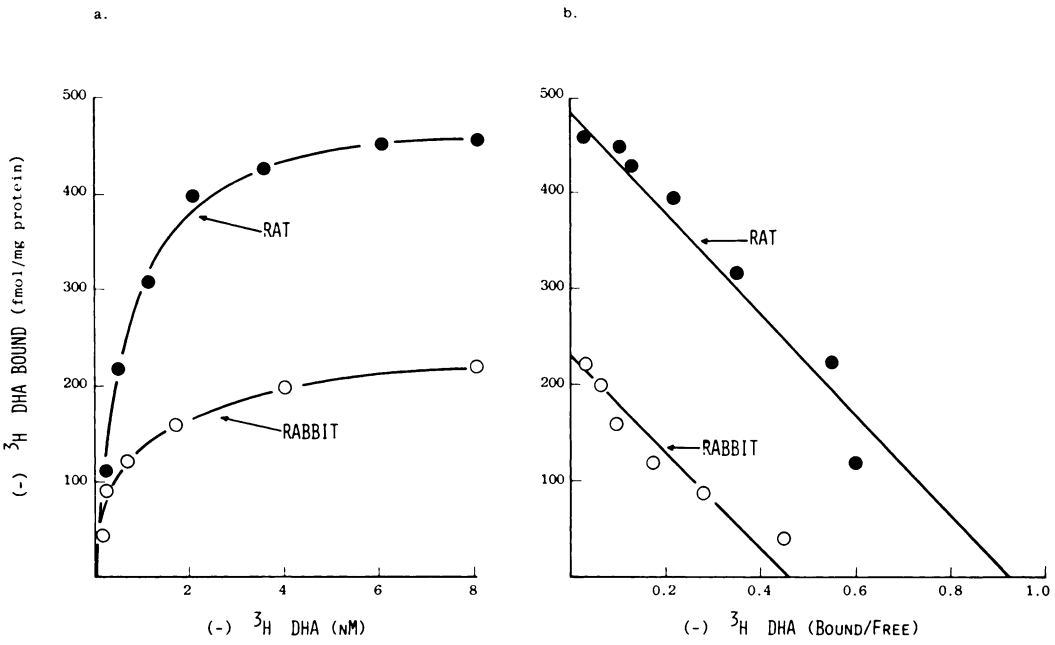

FIG. 1. *Binding of [^{3}H]dihydroalprenolol to lung membrane of rat and rabbit*

a. Specific binding of [^{3}H]dihydroalprenolol to lung membranes prepared from rat (●) or rabbit (○). Specific binding is defined as the difference between total binding and the binding in the presence of 200 μM (−)isoproterenol. b. Scatchard analysis of the binding curves. The data shown are those from a single experiment performed in triplicate. Scatchard analysis of the binding curves from three separate experiments yielded the following results. Rat lung K_d = 0.50 ± 0.11 nM (mean ± S.E.M.); B_{max} = 450 ± 30 fmol/mg protein. Rabbit lung K_d = 0.41 ± 0.03 nM; B_{max} = 230 ± 10 fmol/mg protein.

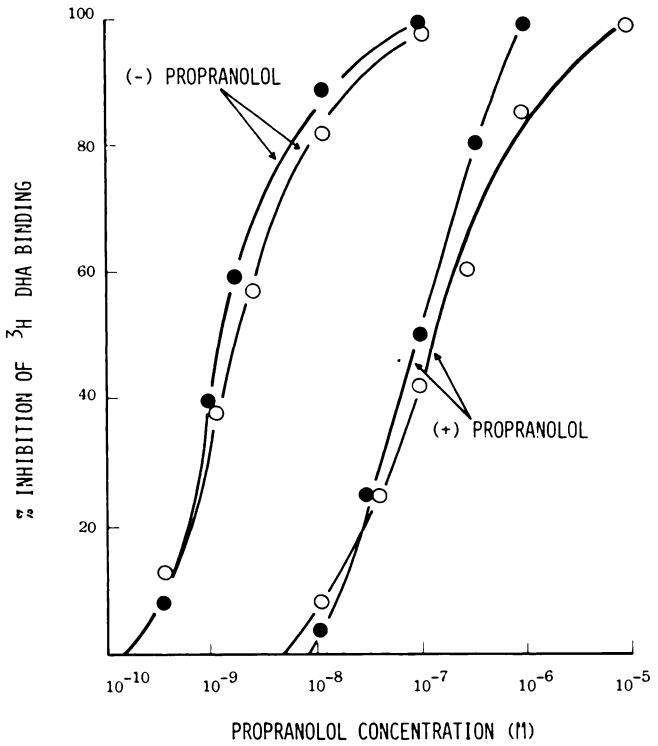

FIG. 2. *Inhibition of specific [^{3}H]dihydroalprenolol binding to rat (●) or rabbit (○) lung membranes by (−)propranolol and (+) propranolol*

The data shows the mean of 3–4 experiments performed in duplicate and the S.E.M. of each point was < ±5%.

hibited the pharmacological characteristics of the *beta* adrenoceptor.

The potency of *beta* adrenergic agonists in displacing [^{3}H]DHA was different in the two species (Fig. 3). Thus in rat lung isoproterenol > epinephrine > norepinephrine, whereas in rabbit lung epinephrine and norepinephrine were equipotent. In the light of the original observations of Lands *et al.* (1), these findings suggest that the rat lung exhibits characteristics of a $beta_2$ receptor whereas, the rabbit may be classified as $beta_1$. These suggestions are supported by the relative affinities of agonists and antagonists that have been reported to show $beta_1$ or $beta_2$ selectivity in intact preparations. Thus, practolol, atenolol and para-oxprenolol ($beta_1$ selective) (2) are all considerably more potent overall in displacing [^{3}H]DHA binding from rabbit than rat lung. In contrast, however, salbutamol, a $beta_2$ selective agonist (3) possessed only slightly higher affinity for the rat membrane sites, whereas the new highly $beta_2$-selective bronchodilator, (−)erythro procaterol (OPC-2009) (13), possessed more than a 20-fold greater affinity for rat lung *beta* adrenoceptor binding sites (Table 1).

It was noticed that the overall inhibition curves for the highly selective agents ($beta_1$ and $beta_2$) had a low slope, as compared with those of the nonselective agents. For example, timolol (nonselective) produces a steep displacement curve (Fig. 4a) identical in both species. The Scatchard analysis (Fig. 4b) of this data is best described by a single straight line, the slope of which represents the IC_{50} (concentration of agent causing 50% displacement of specific [^{3}H]-DHA binding) for this compound. On the other hand, the displacement curves for practolol (Fig. 5a) in both species are different from the timolol curves. Thus, the rat lung practolol curve has a distinct inflection at lower concentrations of antagonist, while the rabbit lung curve shows that

TABLE 1

K_i values (inhibition constants) for beta *adrenergic agonists and antagonists determined from the overall displacement curves of [^{3}H]-dihydroalprenolol from rat and rabbit lung membranes*[a]

Agent	Rat	Rabbit
	K_i (M)[b]	
(−)Isoproterenol	7.2×10^{-8}	4.2×10^{-8}
(−)Epinephrine	3.3×10^{-7}	4.2×10^{-7}
(−)Norepinephrine	3.3×10^{-6}	6.5×10^{-7}
(±)Salbutamol	1.4×10^{-6}	3.0×10^{-6}
(−)OPC-2009	3.5×10^{-8}	7.6×10^{-7}
(−)Propranolol	3.9×10^{-10}	7.8×10^{-10}
(+)Propranolol	3.2×10^{-8}	3.4×10^{-8}
(−)Timolol	6.2×10^{-10}	6.3×10^{-10}
(±)Oxprenolol	1.1×10^{-9}	2.5×10^{-9}
(±)Practolol	2.8×10^{-5}	1.3×10^{-6}
(±)Atenolol	6.7×10^{-6}	4.5×10^{-7}
(±)Para-oxprenolol	1.4×10^{-5}	1.0×10^{-6}

[a] In all experiments [^{3}H]DHA bound was <10% of the total added.

[b] K_i is determined from the equation $K_i = IC_{50} \Big/ \left(1 + \frac{S}{K_d}\right)$, where S = concentration [^{3}H]DHA in the assay (1–2 nM) and K_d = 0.5 nM. + The data are the means of 3–4 separate experiments performed in duplicate using a minimum of 6 concentrations of each agent (S.E.M. in each case <10%).

practolol is more potent in this species and that at higher concentrations the slope is low with distinct flattening. OPC-2009 (Fig. 5b) reversed the appearance of the curves. This agent has higher affinity for rat than for rabbit membranes, and the latter displacement curve now shows an inflection at low concentrations of drug.

Scatchard analysis of the data with these selective agents does not indicate a simple interaction with a single population of homogeneous receptors. In previous analysis with rat lung membranes (10) we have argued against cooperative interactions and suggested that the nonlinear Scatchard plot indicates heterogeneity of binding sites. In the present work strong support for this suggestion comes from the comparisons of the two species with both *beta*$_1$ and *beta*$_2$ agents. Scatchard plots can be best described by two distinct straight lines for each selective agent, indicating the presence of two binding sites, one of low affinity (A) and the other of high affinity (B), for each compound, (Fig. 6a and 6b). Extrapolation of each line on the Scatchard plot to the y axis gives a measure of the relative proportions of each binding site, which were different in each species. Thus the proportion of high affinity sites for practolol (*beta*$_1$) was 25% and 60% in rat and rabbit lung, respectively. OPC-2009 (*beta*$_2$) produced an exact mirror image of this situation within each species. Thus the proportions of high affinity sites for OPC-2009 were 75% and 40% in rat and rabbit respectively. The IC_{50}s and K_i values calculated for the slopes of each component (A and B) for both compounds showed excellent correlation between the species (Fig. 6 and Table 2).

Similar analysis was performed with other agents that exhibited the greatest selectivity between the two species (atenolol, para-oxprenolol). In all cases similar proportions of *beta*$_1$ and *beta*$_2$ binding sites were indicated and close correlation was again found between the two Scatchard components (A and B) for each compound between the two species (Table 2).

DISCUSSION

The characteristics of [^{3}H]DHA binding to rat and rabbit lung membranes exhibit properties that have been previously observed in particulate preparations prepared from a number of mammalian tissues (14, 15). Thus a large proportion of the binding was of high affinity, rapid, and reversible, stereoselective and was displaced by adrenergic agents in a manner suggesting that the labeled sites are probably the cell surface recognition sites of the *beta* adrenoceptor.

Much pharmacological data in intact tissue preparations has indicated that the *beta* receptor in the trachea and lung possess characteristics that differ sufficiently from those in the heart to indicate a clear differentiation of the receptors, though the strict subdivision into *beta*$_1$ and *beta*$_2$ is still the subject of some debate (2–5). However, until recently the characterization of the *beta* adrenoceptor has only been assessed by indirect pharmacological means and is clearly subject to certain limitations. Thus, for example, it might be expected that the tissue distribution of agents that possess different physicochemical properties could

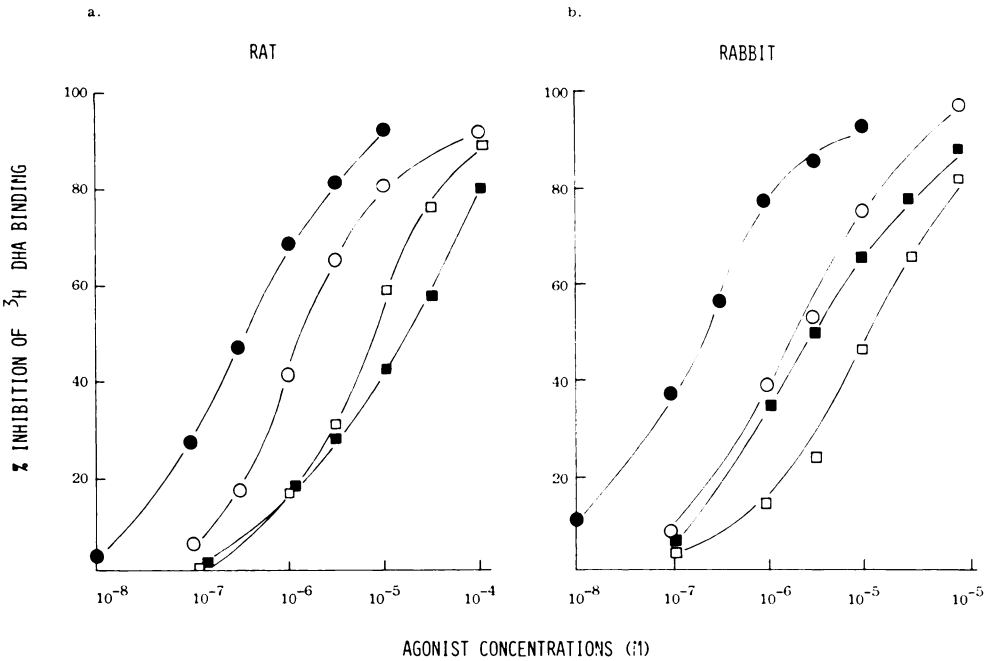

FIG. 3. *Inhibition of specific [^{3}H]dihydroalprenolol binding to rat (a) or rabbit (b) lung membranes by beta adrenoceptor agonists.*

Isoproterenol (●——●), epinephrine (○——○), norepinephrine (■——■), and salbutamol (□——□). The data shows for each agonist the mean of 3–4 experiments performed in duplicate and the S.E.M. of each point was < ±5%.

vary substantially from organ to organ. Thus, differing rates of equilibrium of drugs between bulk and receptor phases could give the false impression of a heterogeneous population of *beta* adrenoceptors. On the other hand, support for a true heterogeneity of sites has come from biochemical approaches. Both Burges and Blackburn (16) and Lefkowitz (17) demonstrated that the *beta* adrenoceptor coupled to the adenylate cyclase in rat and canine lung membranes differed pharmacologically from that in the heart. Coleman and Somerville (12) however, provided recent evidence that rabbit lung *beta* receptors coupled to adenylate cyclase surprisingly demonstrated an overall $beta_1$ response.

In our previous preliminary studies (10), which were the first to examine lung *beta* adrenoceptors by the direct binding of a labeled ligand, we clearly demonstrated that the pharmacological characteristics of rat lung membrane sites differed ($beta_2$) characteristically from those in the heart ($beta_1$). In the present study we provide evidence that the *beta* adrenoceptor binding sites in rabbit lung, unlike those in rat or indeed pulmonary tissue from several other species (unpublished results), possess properties more akin to $beta_1$ adrenoceptors. This is clearly reflected by the relative potencies of selective *beta* adrenergic agonists and antagonists. Thus, it seems that the conclusions concerning the nature of the pulmonary *beta* receptor reached by Coleman and Somerville (12) probably reflect an unfortunate choice of species for their experiments.

In the present study, not only is there clear evidence of a differentiation between the *beta* adrenoceptor in rat and rabbit lung, but it also seems probable that the difference may reflect varying proportions of the two *beta* adrenoceptor subtypes within each tissue. In previous experiments, we had noticed that the displacement of [^{3}H]DHA binding to lung membranes by highly selective $beta_1$ agents was not characteristic of interaction with a single homogeneous population of receptors (10).

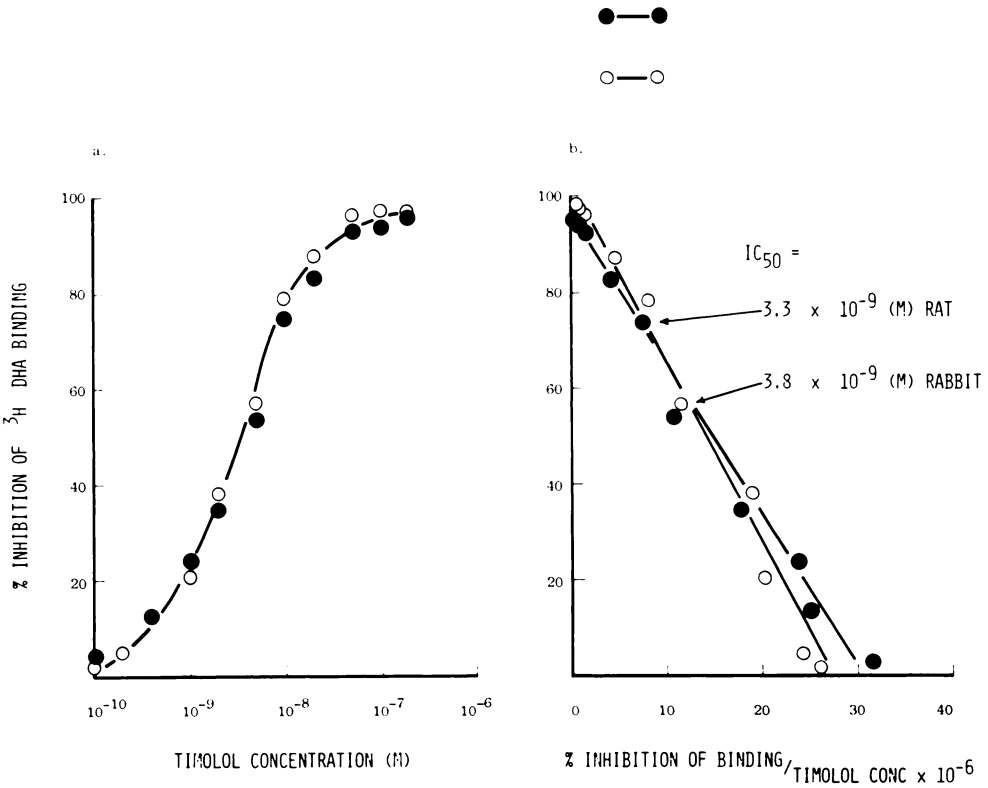

FIG. 4. *Inhibition of [^{3}H]dihydroalprenolol binding to rat and rabbit lung membrane by timolol*

a. Inhibition of specific [^{3}H]dihydroalprenolol binding to rat (●) or rabbit (○) lung membranes by the nonselective *beta* adrenoceptor antagonist (−)timolol. b. Scatchard analysis of the inhibition curves for both species. The data show in each case the mean of 3–4 experiments performed in duplicate; the S.E.M. of of each point was < ± 5%. IC_{50}'s as indicated in the figure are defined as the concentration of agent producting 50% inhibition of specific [^{3}H]DHA binding.

This finding has been confirmed in the present study with both rat and rabbit lung. Careful analysis of the displacement curves in the present work by Scatchard analysis indicates that although the major proportion of sites in rat lung are *beta*$_2$ (75%) and those in rabbit are *beta*$_1$ (60%), both tissues possess a heterogeneous mixture of both *beta* adrenoceptor subtypes. Conclusive evidence that these binding sites were truly related to *beta*$_1$ and *beta*$_2$ adrenoceptors required analysis with a highly selective *beta*$_2$ agent. It was, however, surprising that agents such as salbutamol, which have considerably greater effects in tissues possessing *beta*$_2$ receptors (3), exhibited only slightly different affinities in the two species. It seems possible that salbutamol and other compounds that have substitutions in the phenyl ring may exert their selectivity by virtue of their efficacy rather than by their affinity for the receptor sites. Certainly salbutamol can stimulate adenylate cyclase in rat lung, though it is relatively ineffective in heart (16) and in rabbit lung (12). However, following an extensive examination of the structure-affinity relationships for *beta*$_2$ adrenoceptors (in preparation), it became obvious that agents with substitutions at the *alpha* carbon of the side chain all show selective affinity toward the *beta*$_2$ adrenoceptor. One of these compounds (OPC-2009), recently shown to be a potent and highly selective bronchodilator (13), has proved critical in our analysis. This agent exhibited a 20-fold higher affinity for rat than rabbit lung overall. Scatchard analysis of displacement curves using

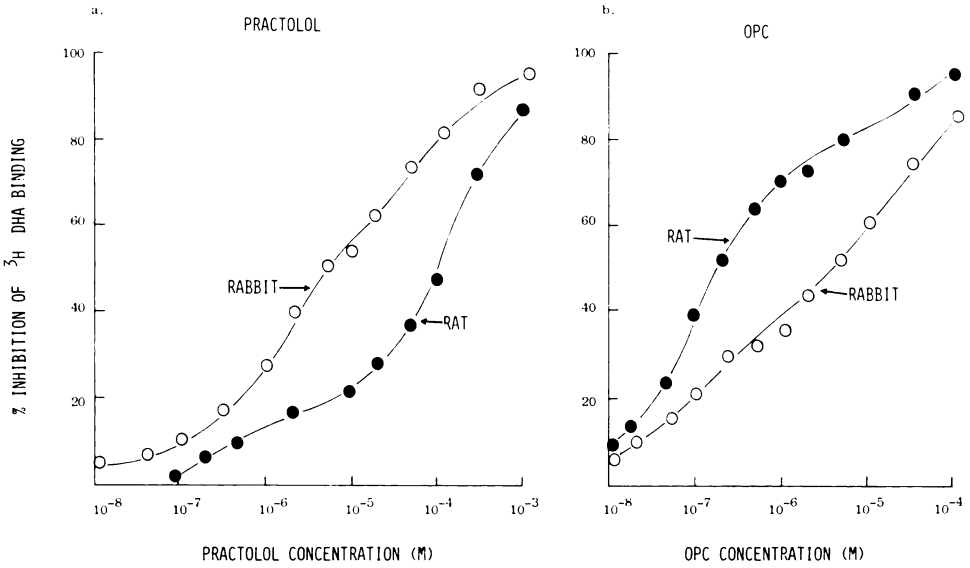

FIG. 5. *Inhibition of specific [^{3}H]dihydroalprenolol binding to rat (●) or rabbit (○) lung membranes by the selective beta$_1$ antagonist practolol (a) or the selective beta$_2$ agonist OPC-2009 (b)*

The data show in each case the mean of 3–4 experiments performed in duplicate and the S.E.M. of each point was < ±5%.

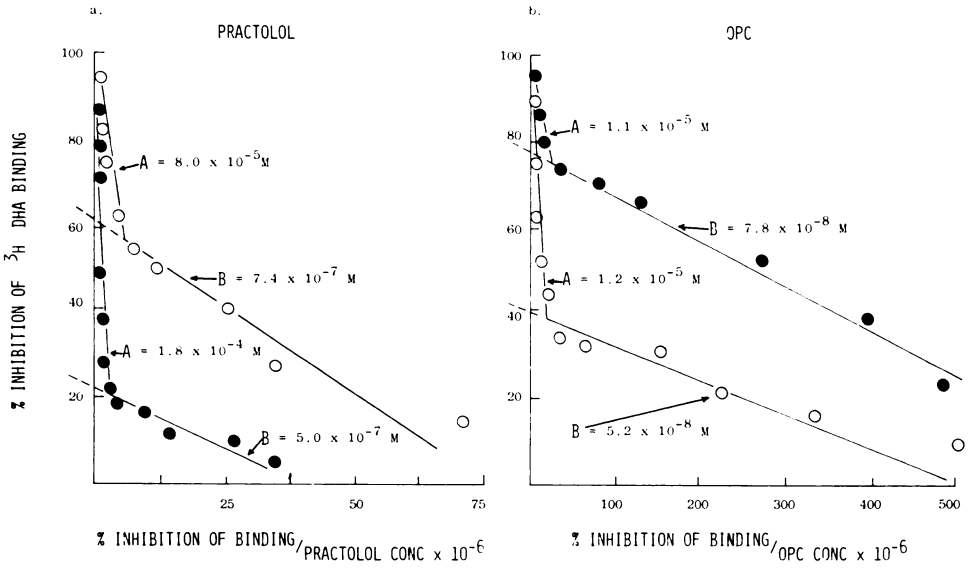

FIG. 6. *Scatchard analysis of the inhibition curves illustrated in Fig. 5 for practolol (a) and OPC-2009 (b) from rat (●) and rabbit (○) lung membranes*

IC$_{50}$s for each binding site component (A, high affinity; B, low affinity) are indicated in the figure (S.E.M. < ±10%, n = 3 or 4 experiments). Components A and B were calculated by standard linear regression techniques. The point of inflection between the lines was arbitrarily decided when the high affinity component (line B) giving the highest correlation coefficient was calculated. Using the y axis intercept for line B the true value for the low affinity component line A was calculated by subtracting this intercept value from 100% and expressing each point on line A as a percentage of the remaining proportion. For clarity, lines A in the figure are drawn uncorrected but IC$_{50}$s represent corrected values.

TABLE 2

K_i values (inhibition constants) for the selective beta *adrenergic agents determined for each component (A and B) of the Scatchard analysis*

Agent	Scatchard Component	Rat[b]	Rabbit
		K_i (M)[a]	
Practolol	A	4.3×10^{-5} (74)	1.3×10^{-5} (46)
(β_1)	B	2.0×10^{-7} (23)	1.4×10^{-7} (53)
Atenolol	A	1.8×10^{-5} (79)	9.0×10^{-6} (36)
(β_1)	B	9.0×10^{-8} (26)	8.1×10^{-8} (61)
Para-oxprenolol	A	1.3×10^{-5} (74)	1.8×10^{-5} (39)
(β_1)	B	2.7×10^{-7} (25)	2.8×10^{-7} (62)
OPC-2009	A	2.4×10^{-6} (22)	2.3×10^{-6} (61)
(β_2)	B	1.7×10^{-8} (76)	1.0×10^{-8} (36)

[a] K_i is defined as in Table 1. Data for both species are determined from the slope (IC_{50}) of each Scatchard component and are the means from 3–4 experiments performed in duplicate using 10–12 concentrations of agent in each case (S.E.M. < 10%).

[b] Values in brackets are the proportion of each binding site (%) derived from extrapolation by linear regression of lines A and B to the y axis (see Fig. 6). The mean percentages (± S.E.M) were: rat β_1 = 24.0 ± 0.9%, β_2 = 75.6 ± 1.2%; rabbit β_1 39.3 ± 2.4%, β_2 59.0 ± 2.0%.

this compound produced a "mirror image" picture to the *beta*$_1$ selective drugs in both species, providing strong confirmatory evidence concerning the proportions of *beta*$_1$ and *beta*$_2$ adrenoceptor binding sites in rat and rabbit lung.

Interestingly, although the relative proportions of the two *beta* adrenoceptor subtypes are different between the two species, the absolute concentration of the "*beta*$_1$" component is fairly constant in both sets of membranes (113 and 138 fmol/mg protein in rat and rabbit lung, respectively). The concentration of "*beta*$_2$" binding sites is considerably lower in the rabbit (92 fmol/mg protein) as compared to the rat (337 fmol/mg protein) and accounts for the lower total concentration of binding sites found in the rabbit membranes (Fig. 1). Clearly future investigations must be directed toward the physiological significance of these findings. It is possible that the heterogeneous nature of *beta* adrenoceptors in lung may only reflect the heterogeneous nature of the tissue. However, evidence is accumulating that both *beta*$_1$ and *beta*$_2$ receptors may be involved in cardiac stimulation (7) and in tracheal smooth muscle relaxation (8). However, since there is evidence that rabbit airways smooth muscle is relatively insensitive to isoproterenol (4, 11) it is tempting to relate this to the lower proportion of *beta*$_2$ adrenoceptors in this tissue and to suggest that it is this subtype that mediates pulmonary smooth muscle relaxation. At the present time we are attempting to obtain more definitive evidence on this point by using cell separation techniques and by the use of the recently developed fluorescent probes for *beta* adrenoceptors (18).

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