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Homogeneity of Beta$_2$-Adrenoceptors on Rat Erythrocytes and Reticulocytes

A Comparison with Heterogeneous Rat Lung *Beta*-Adrenoceptors

K. DICKINSON, A. RICHARDSON,[1] AND S. R. NAHORSKI

Department of Pharmacology and Therapeutics, University of Leicester, Leicester, United Kingdom

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SUMMARY

DICKINSON, K., A. RICHARDSON, AND S. R. NAHORSKI. Homogeneity of *beta*$_2$-adrenoceptors on rat erythrocytes and reticulocytes: a comparison with heterogeneous rat lung *beta*-adrenoceptors. *Mol. Pharmacol.* 19:194–204 (1981).

The characteristics of specific ^{3}H-labeled (−)-dihydroalprenolol ([^{3}H]DHA) binding sites on rat erythrocytes and reticulocytes have been examined in an attempt to determine whether different *beta*-adrenoceptor subtypes could coexist in the same population of cells. [^{3}H]DHA binds specifically to erythrocyte membranes with a dissociation equilibrium constant (K_D) of 0.24 nM and a maximal number of binding sites (B_{max}) of 195 fmoles/mg of protein. Reticulocytes induced by phenylhydrazine treatment possessed a considerably higher density of binding sites (B_{max} 760 fmoles/mg of protein) but identical affinity for [^{3}H]DHA (K_D 0.25 nM).

A detailed pharmacological characterization using highly selective *beta*$_1$- and *beta*$_2$-adrenoceptor agents revealed that both cell populations possessed only homogeneous *beta*$_2$-adrenoceptor binding sites that were pharmacologically identical with the *beta*$_2$ component observed in rat lung which possesses both *beta*$_1$- and *beta*$_2$-adrenoceptors. Further evidence for the heterogeneous nature of *beta*-adrenoceptor binding sites in rat lung was provided by the demonstration that selective occupation of the *beta*$_1$ sites with atenolol results in a loss of receptor heterogeneity, and suggests that the receptor subtypes are probably separate noninteracting entities. Methods to analyze heterogeneous binding data (Hofstee and Hill plots of transformed data and computer-assisted iterative curve-fitting of the raw data) were compared and indicate that, with highly selective competing drugs, linear transformations provide data in excellent agreement with those obtained with curve-fitting.

INTRODUCTION

The recent development of ligand-binding techniques using radiolabeled *beta*-adrenergic antagonists has allowed the direct quantitative characterization of *beta*-adrenoceptors in tissues. Direct receptor labeling techniques (using drugs with high selectivity for *beta*$_1$- and *beta*$_2$-adrenoceptors) have been applied by our laboratories to demonstrate the coexistence of *beta*-adrenoceptor subtypes in rat lung, brain, and spleen (1–3). The present studies were undertaken to determine whether different *beta*-adrenoceptor subtypes could coexist in the same population of cells, and we have selected rat erythrocytes as a convenient source of single-cell types which contain *beta*-adrenoceptors.

Rat erythrocytes and reticulocytes have been useful model cells for studying *beta*-adrenoceptor-effector systems (4), and comparative studies of reticulocytes and erythrocytes have provided information on the coupling of the *beta*-adrenoceptor to adenylate cyclase (5–8). The *beta*-adrenoceptor of these cells has previously been classified, using adenylate cyclase measurements (9) and ligand-binding techniques (5, 8), as *beta*$_2$-adrenoceptors. However, such a classification differed from that suggested for the reticulocyte *beta*-adrenoceptor by Gauger *et al.* (10), with evidence not altogether dissimilar to that above, but lacking the use of highly selective antagonists.

We report here our studies designed to investigate whether this apparent discrepancy of *beta*-adrenoceptor classification is due to the heterogeneous nature of these receptors on rat erythrocytes and reticulocytes. The affinities of several *beta*-adrenoceptor selective drugs de-

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[1] Present address, Department of Obstetrics and Gynaecology, Ninewells Hospital and Medical School, University of Dundee, Dundee, Scotland DDI 9SY.

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termined in competition with specific [^{3}H]DHA[2] binding to erythrocyte and reticulocyte *beta*-adrenoceptors have been compared with those determined with rat lung *beta*-adrenoceptors in which recent studies have shown the presence of a heterogeneous population of receptors pharmacologically equivalent to $beta_1$- and $beta_2$-adrenoceptor subtypes (1, 11, 12). Our findings suggest that the adrenoceptors present on reticulocytes and erythrocytes are homogeneous and identical with the $beta_2$-adrenoceptor component present in rat lung.

MATERIALS AND METHODS

Preparation of membranes. Red cell membranes were prepared from control (0.9% NaCl-injected) and phenylhydrazine-treated male Wistar rats (150–200 g). Reticulocyte-rich blood was obtained following i.p. injection of a sodium bicarbonate-neutralized solution of phenylhydrazine HCl according to the following dosing regimen: day 1, 40 mg/kg; day 3, 60 mg/kg; day 5, 80 mg/kg. This procedure produced a red cell population which was >90% reticulocytes. Rats were killed on day 8, and blood (8 ml) was obtained by cardiac puncture under sodium pentobarbital anesthesia (50 mg/kg) using 0.6 ml of 0.2 M EDTA as an anticoagulant. The blood was centrifuged at 500 × *g*, the plasma was removed by aspiration, and the cells were washed in cold isotonic 150 mM NaCl/5 mM Tris-HCl, pH 7.6, and recentrifuged at 500 × *g*. The supernatant was removed and the cells were washed three more times in 150 mM NaCl/5 mM Tris-HCl, pH 7.6, by centrifugation at 1,500 × *g*, after which a buffy-coat layer was no longer visible. Aliquots (1 ml) of packed cells were removed from the bottom of the centrifuge tube and lysed in 40 ml of 5 mM Tris-HCl, pH 7.8. After 10 min at 4°, 1 ml of 4 M KCl-40 mM $MgCl_2$ was added and the lysate was centrifuged at 50,000 × *g* for 30 min at 4°. The pellet was resuspended in 5 mM Tris-HCl, pH 7.8, and relysed. After centrifugation at 50,000 × *g* for 30 min, the upper plasma membrane-enriched layer was removed and washed three more times in 50 mM Tris-HCl, pH 7.8, by centrifugation at 50,000 × *g*. The final pellet was taken up in 50 mM Tris-HCl, pH 7.8, at a protein concentration of 3–5 mg/ml, rapidly frozen in liquid nitrogen, and stored at −40° until use. Freezing did not affect the maximal specific binding of [^{3}H]DHA to the membranes.

Rat lung membranes were prepared from male Wistar rats (150–200 g) as previously described (1).

Binding assay. Membrane suspensions were incubated at 23° with [^{3}H]DHA (48–52 Ci/mmole; New England Nuclear Corporation, Boston, Mass.), approximately 1 nM unless otherwise stated, and appropriate concentration of drug in 50 mM Tris-HCl, pH 7.8, in a final incubation volume of 250 μl. After 30 min the samples were diluted with 1 ml of ice-cold buffer and filtered under reduced pressure through Whatman glass fiber discs (GF/B); the filters were then washed three times with buffer, 5 ml each time. The filters were shaken in the cold with Triton X-100-toluene scintillator and, after extraction, the radioactivity was determined by liquid scintillation counting at an efficiency of 40%.

"Specific" binding was defined as total binding minus "nonspecific" binding. Nonspecific binding was determined by measuring the radioactivity obtained when incubations were carried out in the presence of 200 μM (−)-isoprenaline. We have previously shown that the use of *beta*-adrenoceptor antagonists to determine nonspecific binding can result in considerable errors in evaluations of apparent dissociation constants and maximal number of binding sites (13). Specific binding of [^{3}H]-DHA to red blood cell and lung memranes constituted approximately 95% and 90%, respectively, of the total binding at 1 nM [^{3}H]DHA.

Specific binding was a linear function of protein concentration up to 250 μg of protein for all membrane preparations at 1 nM [^{3}H]DHA. Conditions were chosen, in all experiments, such that the total bound [^{3}H]DHA was less than 10% of the total [^{3}H]DHA added, thereby ensuring that the "free" component of [^{3}H]DHA was relatively constant.

Determination of protein. Protein concentrations were measured according to the method of Lowry *et al.* (14), using bovine serum albumin as reference standard.

Analysis of binding data. All experiments were conducted a minimum of three times. Equilibrium dissociation constants (K_D) and binding site maxima (B_{max}) were determined by Scatchard plots. IC_{50} values for the displacement of [^{3}H]DHA binding were determined from Hill plots, and inhibition constant (K_i) values were determined using the equation of Cheng and Prusoff (15). Association and dissociation rate constants were determined by graphical analysis of kinetic data using the method described by Williams *et al.* (16).

Inhibition curves of drug displacement of [^{3}H]DHA were fitted on a Cyber 73 computer using an iterative curve-fitting computer program (NAG library, E04FBF, NAG Limited, Oxford, England, 1977), arriving at the best-fit parameters by minimization of the sum of squares. One or two noninteracting site models were tested for each inhibition curve.

Drugs. [^{3}H]DHA (48–52 Ci/mmole) was obtained from New England Nuclear Corporation. (−)-Isoprenaline bitartrate, (−)-adrenaline bitartrate, and (−)-noradrenaline bitartrate were purchased from Sigma Chemical Company, St. Louis, Mo. The following drugs were kindly donated by the indicated companies: (±)-Salbutamol (Glaxo Allenbury's, Ware, England); (−)-timolol (Leo Laboratories, Middlesex, England); (−)-alprenolol (Haessle, Goteborg, Sweden); (±)-procaterol (Otsuka, Tokushima, Japan); (±)-metoprolol (Astra, Goteborg, Sweden); (±)-atenolol, (±)-practolol, (+)-propranolol, (−)-propranolol, and (±)-ICI 118.551 [erythro-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol] (ICI Pharmaceuticals, Cheshire, England); (±)-SL 75212 (betaxolol) [1-(isopropylamino)-3-(*p*-(cyclopropylmethoxyethyl)-phenoxy)-2-propanol] (Synthelabo, Paris, France).

RESULTS

[^{3}H]DHA Binding to Rat Erythrocyte and Reticulocyte Membranes

Increasing concentrations of [^{3}H]DHA were incubated with the membranes in the absence and presence of 200

[2] The abbreviation used is: [^{3}H]DHA, ^{3}H-labeled dihydroalprenolol.

μM (−)-isoprenaline to assess nonspecific binding. Specific binding of [^{3}H]DHA to rat erythrocyte and reticulocyte membranes (Fig. 1) was saturable, whereas nonspecific binding increased linearly with [^{3}H]DHA concentration. Scatchard analysis of such specific binding data revealed a homogeneous population of binding sites with a K_D of 0.24 (± 0.03) nM (n = 5) for rat erythrocyte membranes and 0.25 (± 0.02) mM (n = 3) for rat reticulocyte membranes. Clearly, from Fig. 1, although the affinities of [^{3}H]DHA for the binding sites in rat erythrocytes and reticulocytes were similar, the total number of binding sites was 3 to 4-fold higher in reticulocytes. The mean B_{max} was 760 ± 9) fmoles/mg of protein (n = 3) for reticulocytes and 195 (± 8) fmoles/mg of protein (n = 5) for erythrocytes. Analysis of these saturation data by the method of Hill revealed an absence of cooperative interactions, since the Hill coefficient (n_H) equaled 0.98 for erythrocytes and 0.97 for reticulocytes.

Kinetics of [^{3}H]DHA Binding to Rat Blood Cell Membranes

The binding of [^{3}H]DHA to rat erythrocytes was rapid and reversible (Fig. 2) with a $t_{1/2}$ (time for half-maximal binding) for association of 1.75 min and dissociation of 15 min. Since the concentration of ligand used in these experiments (1.2 nM) was much greater than the binding site concentration (0.11 nM), the association could be considered a pseudo-first order reaction. The reaction can be described by the equation $\ln[X_{eq}/(X_{eq} - X)] = K_{obs} \cdot t$, where X is [^{3}H]DHA bound at time t, and X_{eq} is the amount bound at equilibrium. The slope of the line in Fig. 2A equals the observed rate constant for the reversible pseudo-first order reaction (K_{obs}). The second-order association rate constant K_{+1} was calculated from $K_{+1} = K_{obs} - K_{-1}/([^3H]DHA)$, where K_{-1} is the dissociation rate constant. The dissociation rate constant K_{-1} was calculated from the first-order plot $K_{-1} \cdot t = \ln X/X_{eq}$. Using [^{3}H]DHA concentrations of 1.2–1.4 nM, a mean K_{+1} of 0.22 (± 0.03) nM^{-1} min^{-1} was obtained at 23° (n = 3). The mean K_{-i_1} was 0.047 (± 0.01) min^{-1} (n = 3). The dissociation constant calculated from the fraction K_{-1}/K_{+1} was 0.21 nM, in excellent agreement with that obtained from equilibrium experiments.

Using identical techniques, the binding of [^{3}H]DHA to rat reticulocyte membranes yielded a value for K_{+1} of 0.197 (± 0.014) nM^{-1} min^{-1} (n = 3), for K_{-1} of 0.068 (± 0.003) min^{-1} (n = 3), and a K_D of 0.34 nM.

Pharmacological Characterization of the [^{3}H]DHA Binding Sites

Agonists. Competition experiments in which adrenergic agonists and antagonists were used to displace the specific binding of [^{3}H]DHA were performed in order to characterize the [^{3}H]DHA binding sites of rat erythrocytes and reticulocytes. The affinities of adrenergic agonists for the *beta*-adrenoceptor sites of rat erythrocytes and reticulocytes were compared with those determined using rat lung membranes (Fig. 3). From these data it is

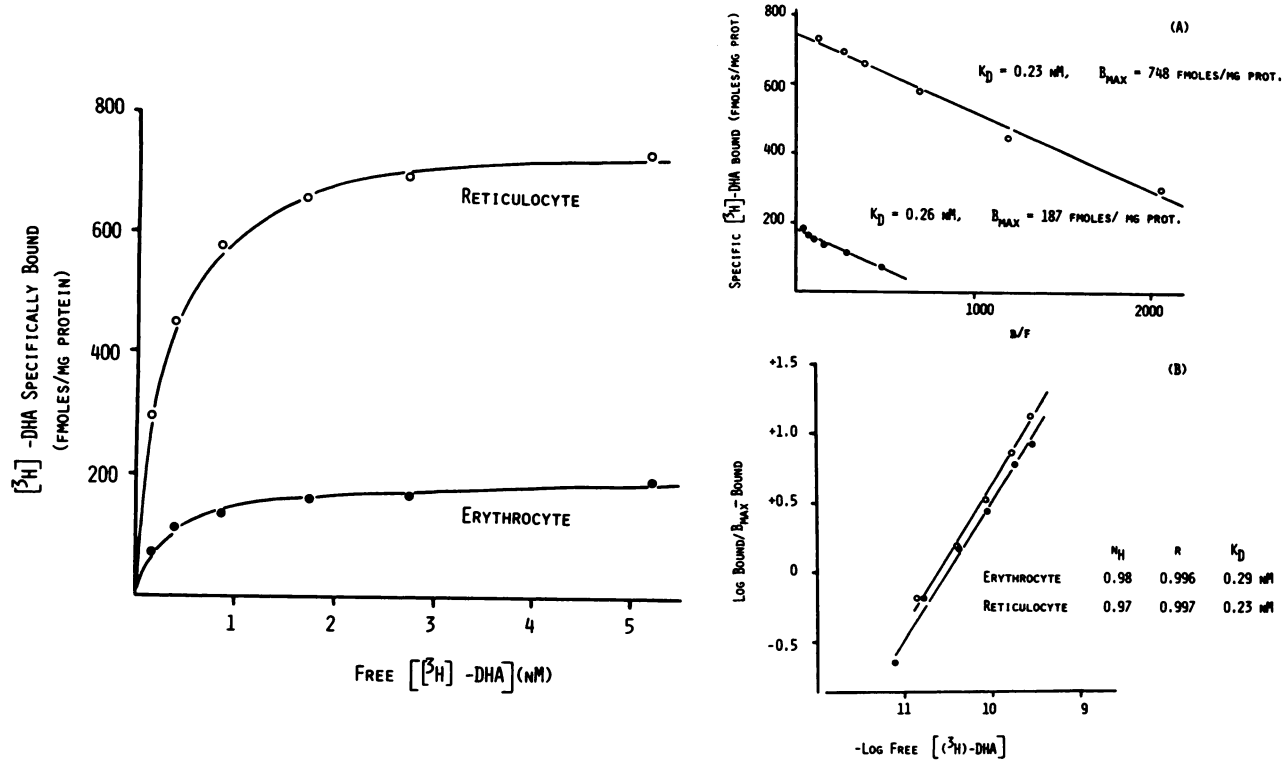

FIG. 1. *Specific binding of [^{3}H]DHA to rat erythrocyte (●) and rat reticulocyte (○) membranes*

Rat blood cell membranes were incubated with increasing concentrations of [^{3}H]DHA for 30 min at 22°, and binding was determined as described under Materials and Methods. Each value is the mean of triplicate determinations. The data shown are representative of five such experiments.

A. Scatchard plot of [^{3}H]DHA binding to rat erythrocyte and reticulocyte membranes. K_D is the equilibrium dissociation constant for [^{3}H]DHA and B_{max} is the maximal number of [^{3}H]DHA binding sites.

B. Hill plot of these saturation data. n_H is the Hill coefficient and is equal to the slope of the line.

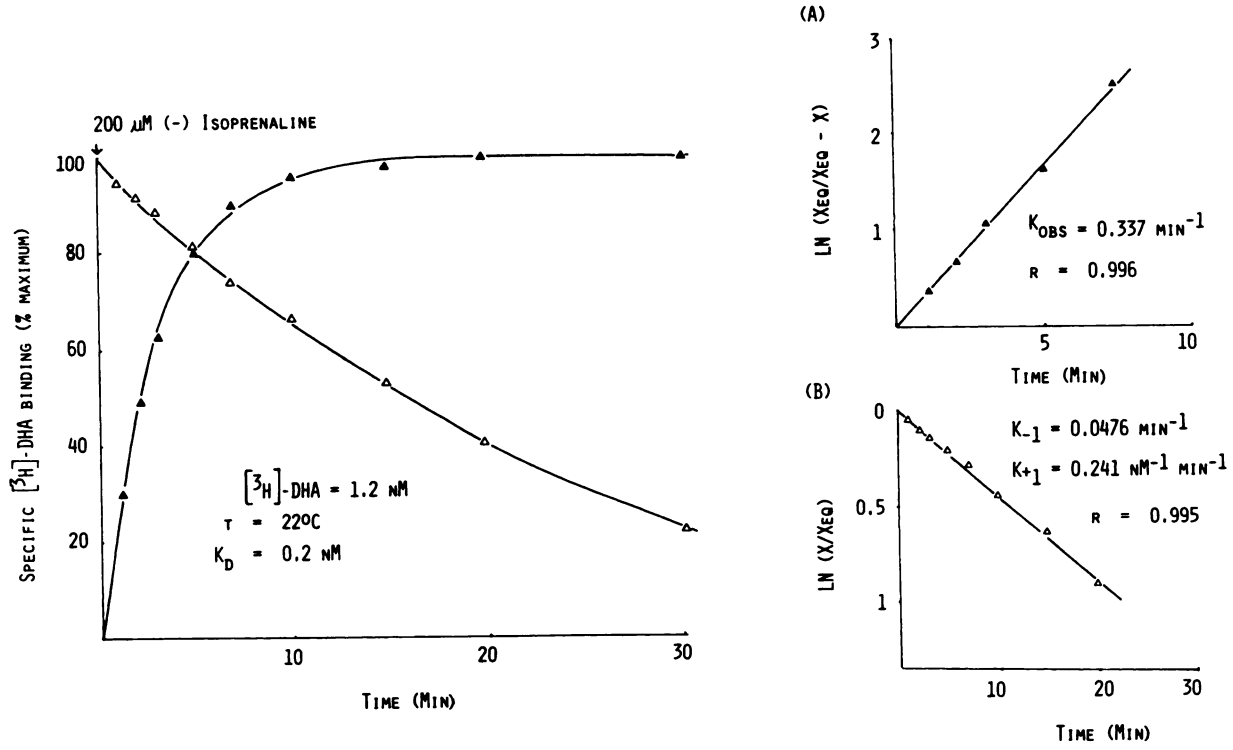

FIG. 2. *Kinetic analysis of [^{3}H]DHA binding to rat erythrocyte membranes*

Membrane preparations were incubated with 1.2 nM [^{3}H]DHA at 22° for various times, and the amount of specific [^{3}H]DHA bound to the membranes was plotted against time (▲). After equilibrium was achieved (30 min at 22°), (−)-isoprenaline was added (200 μM) and the decrease in specific [^{3}H]DHA binding was measured at timed intervals (△). From these data the association and dissociation rate constants were determined as described in the text.

A. The pseudo-first order kinetic plot of [^{3}H]DHA binding, where X_{eq} is the amount of specific binding at equilibrium and X is the amount bound at time t. The slope of this line is the observed rate constant for the reversible pseudo-first order reaction (K_{obs}).

B. The first-order plot of the dissociation reaction, where the slope of the line (K_{-1}) equals the dissociation constant. The binding site concentration for these experiments was 0.11 nM. These data are representative of three such experiments performed in duplicate.

apparent that the affinities of the agonists for each receptor preparation were similar (see also Table 1), and the order of potencies, (−)-isoprenaline > (−)-adrenaline > (±)-salbutamol > (−)-noradrenaline, is suggestive of a *beta*$_2$-adrenoceptor.

The use of agonist affinities to characterize receptors is somewhat hazardous in view of a recent report showing that agonists can induce high affinity states of the *beta*-adrenoceptor, which can be interconverted to low-affinity states by guanyl nucleotides (17). The interaction of guanyl nucleotides with agonist binding to these receptor preparations is at present under investigation.

Antagonists. The displacement of [^{3}H]DHA binding to rat erythrocyte and rat lung membranes by a series of *beta*-adrenoceptor antagonists revealed that the binding was stereoselective, since the (−)-isomer of propranolol was considerably more potent than the (+)-isomer (Table 1). The potencies of selective and nonselective *beta*-adrenoceptor antagonists with rat erythrocyte membranes were compared with those obtained using rat reticulocyte and rat lung membranes. A good agreement of inhibition constants was obtained (Tables 1 and 2) which suggests that the *beta*-adrenoceptors of the rat erythrocyte and reticulocyte are identical and that these receptors are similar to those present on rat lung, where the predominant receptor is of the *beta*$_2$ subtype.

Highly selective *beta*-adrenoceptor antagonists produced displacement curves with rat lung membranes which deviated from law of mass action behavior. The competition curves were shallow, with pseudo-Hill coefficients <1, and inflections were especially marked at low (with *beta*$_1$-selective agents) or high (with *beta*$_2$-selective agents) drug concentrations. Displacement curves of the *beta*$_1$-selective [(±)-atenolol] and *beta*$_2$-selective [(±)-ICI 118.551] antagonists, in competition with [^{3}H]DHA binding to rat lung membranes, have been analyzed using the graphical methods of Hofstee and Hill (Figs. 4 and 5). These agents produced curvilinear Hofstee and nonlinear Hill plots, a finding which is suggestive of heterogeneity of the [^{3}H]DHA binding sites present on rat lung membranes. From the Hofstee plots it is apparent that the membranes contain ≃80% of sites having high affinity for *beta*$_2$-selective agents and ≃20% of sites having affinity for *beta*$_1$-selective agents.

In contrast to the flattened displacement curves of (±)-atenolol and (±)-ICI 118.551 with rat lung membranes, the competition curves of these drugs with rat erythrocyte membranes were indicative of homogeneous receptor population with binding governed by mass action law. The displacement data were subjected to a Hofstee and Hill analysis, and linear plots were obtained (Figs. 4 and 5). Hill coefficients for (±)-atenolol (0.97 ±

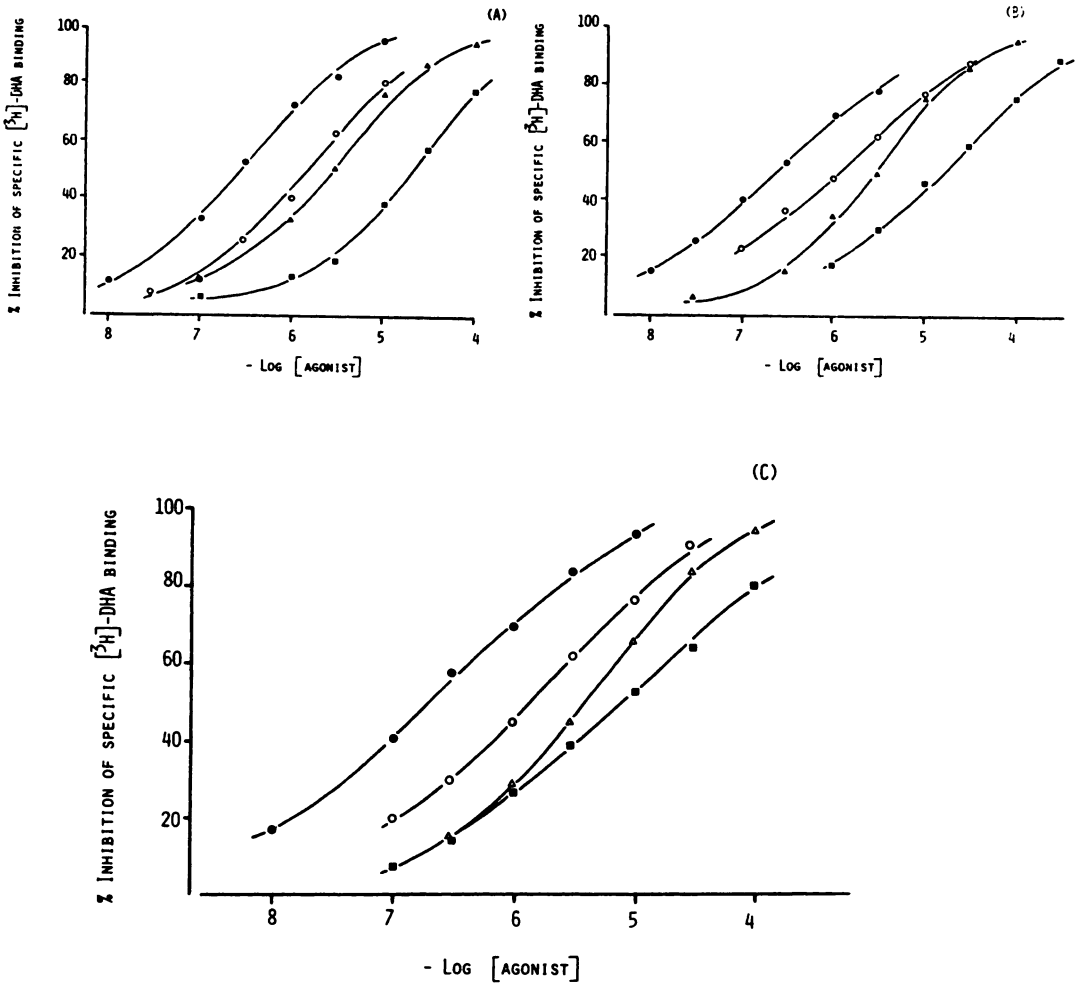

FIG. 3. *Inhibition of specific [^{3}H]DHA binding to rat erythrocyte (A), rat reticulocyte (B), and rat lung (C) membranes by adrenergic agonists*

●, (−)-Isoprenaline; ○, (−)-adrenaline; ■, (−)-noradrenaline; and △, (±)-salbutamol. [^{3}H]DHA, 1.5 nM, and the agonist under investigation [in 50 mM Tris-HCl (pH 7.8) and 1 mM ascorbic acid] were incubated with the membranes for 30 min at 23° and specific binding was assessed as described in the text. The data represent the mean of three to seven experiments conducted in duplicate. Standard errors of the mean were <10% and are excluded for clarity.

0.02, $n = 6$) and (±)-ICI 118.551 (1.03 ± 0.02, $n = 6$) were close to unity. These findings suggest that the [^{3}H]DHA binding sites present on rat erythrocytes are a homogeneous population. Table 2 shows the inhibition constants and Hill slopes of a number of selective agents obtained with rat erythrocyte and rat reticulocyte membranes. Hill slopes of all of these agents approached unity, indicating homogeneity of receptors on rat erythrocytes and reticulocytes.

In addition to the analytical procedures described above, the displacement data for selective adrenoceptor antagonists were fitted to one-site or two-site model concentration-inhibition curves using an iterative curve-fitting computer program. The displacement curves of selective adrenergic agents, obtained in competition with [^{3}H]DHA binding to rat lung membranes, are best fitted to a two-site model, whereas displacement curves of these agents obtained with rat erythrocyte membranes are best fitted to a one-site model. The relative proportion of the two sites in rat lung membranes were 75–83% for the site having high affinity for *beta*$_2$-selective agents (*beta*$_2$-adrenoceptor subtype), and 17–24% for the site having high affinity for *beta*$_1$-selective agents (*beta*$_1$-adrenoceptor subtype) (see Table 2).

Inhibition constants of selective agents obtained with rat erythrocyte and reticulocyte membranes correlated almost perfectly with those obtained for the *beta*$_2$-adrenoceptor component of rat lung (reticulocyte: $r = 0.99$, slope = 0.97; erythrocyte: $r = 0.99$, slope = 1.00) but poorly for the *beta*$_1$-adrenoceptor component. Similar inhibition constants have been obtained for the *beta*$_2$-adrenoceptor component of rat cerebral cortex and spleen (2, 3). These findings argue strongly for the *beta*$_2$-adrenoceptor classification of the rat erythrocyte and reticulocyte *beta*-receptors.

Selective Inhibition of Beta-Adrenoceptor Binding Sites on Lung Membranes

Rat lung membranes were incubated with a concentration of (±)-atenolol (5 μM) which, at 1.5 nM [^{3}H]DHA concentration, was predicted by computer-assisted curve fitting of model curves to occupy 96% of *beta*$_1$ sites and

TABLE 1

Inhibition constants

Effects of *beta*-adrenergic agonists and antagonists on [^{3}H]DHA binding to rat erythrocyte, reticulocyte, and lung membranes. The IC_{50} values were determined graphically and the K_i was calculated from the equation $K_i = IC_{50}/(1 + S/K_D)$, where S is the concentration of [^{3}H]DHA used in the assay (1–1.5 nM) and K_D is the dissociation constant for [^{3}H]DHA in the membrane preparations (rat erythrocyte K_D = 0.24 nM; rat reticulocyte K_D = 0.25 nM; rat lung K_D = 0.35 nM). The affinities of the drugs for rat lung *beta*-receptors are over-all apparent K_i values. The values given are the means ± standard error of the mean of three to eight experiments performed in duplicate.

Drug	Rat erythrocyte	Rat reticulocyte	Rat lung
	nM	*nM*	*nM*
(=)-Isoprenaline	33 ± 3	34 ± 3	27 ± 3
(=)-Noradrenaline	2920 ± 320	2020 ± 280	1430 ± 180
(=)-Adrenaline	220 ± 14	145 ± 13	245 ± 25
(±)-Salbutamol	360 ± 26	371 ± 30	660 ± 180
(±)-Propranolol	29 ± 1.5	=	55 ± 2
(=)-Propranolol	0.44 ± 0.01	=	0.69 ± 0.05
(=)-Timolol	0.39 ± 0.01	0.40 ± 0.03	0.46 ± 0.04
(=)-Alprenolol	0.37 ± 0.02	=	0.54 ± 0.04

14% of *beta*$_2$ sites. The displacement of [^{3}H]DHA binding from the lung membranes by increasing concentrations of the *beta*$_2$-selective partial agonist (±)-procaterol was then determined. Control membranes produced displacement curves with (±)-procaterol which had a distinct inflection at high drug concentrations, and which analyzed as nonlinear Hofstee and Hill plots (Fig. 6). Identical curves were observed in the presence of 100 μM GTP. In contrast, the displacement curve for procaterol in the presence of (±)-atenolol was shifted to the left, and was clearly closer in shape to the theoretical curve expected for a homogeneous receptor population with binding governed by mass-action law. Analysis of these data by the Hofstee plots (1) and iterative curve-fitting revealed the presence of approximately 4% of total specific binding which had low affinity for (±)-procaterol (*beta*$_1$-adrenoceptor), as compared with 29% in control membranes. The Hill plot data (Fig. 6) was a sensitive indicator of the small degree of heterogeneity still present in these membranes (n_H over-all = 0.91). Under these conditions, occupation of *beta*$_1$-adrenoceptor binding sites by 5 μM atenolol had transformed a system with heterogeneous binding sites into a system which was almost homogeneous, and which closely approached that present in rat erythrocytes.

Comparison of Methods to Analyze Binding Data

In previous work we (1, 2, 11) and subsequently others (12) have used the Hofstee plot to analyze competition curves of highly selective *beta*-adrenergic agents in order to determine the proportion of *beta*$_1$ and *beta*$_2$ sites in membrane preparations and the affinities of drugs for each site. The graphical analysis used previously (11) has subsequently been suggested to result in considerable overestimation of the proportion of the high-affinity component, and to introduce considerable error in the IC_{50} values of drugs for the components (18). In order to resolve the possible error involved in previous analyses, the data shown in Figs. 4 and 5 have been analyzed by computer-assisted iterative curve-fitting of the raw data and by graphical extrapolation of Hofstee plots as described previously (1). The results shown in Table 3 clearly demonstrate that the IC_{50} values and proportion of sites obtained upon Hofstee analysis are remarkably similar to those obtained using computer-assisted analysis of the raw data. As expected, the proportion of *beta*$_1$-receptor sites was only very slightly overestimated using the Hofstee analysis. However, as we have indicated previously (1), this method of analysis provides an adequate assessment of the inhibition data only when the drugs used have considerable (>30-fold) selectivity to one receptor subtype. Agents with less selectivity e.g., metoprolol (13- to 22-fold *beta*$_1$-selective) produce less marked curvilinear plots which yield considerable overestimates of the proportion of *beta*$_1$-sites and approximately 3-fold errors in IC_{50} values (Hofstee: *beta*$_1$ 43%, 3.2×10^{-7} M; *beta*$_2$ 64%, 6.5×10^{-6} M; curve-fitting: *beta*$_1$ 17%, 1.2×10^{-7} M; *beta*$_2$ 83%, 2.6×10^{-6} M).

DISCUSSION

The existence of *beta*-adrenoceptor subtypes was first proposed on the basis of relative effector cell responsive-

TABLE 2

Potencies of highly selective adrenergic agents for beta-adrenoceptors of rat reticulocytes, erythrocytes, and lung

K_i values were calculated as described in Table 1. The displacement curves of agents obtained with rat lung membranes were analyzed by an iterative curve-fitting program, and K_i values of drugs for the two sites were determined as described previously. Percentage values in parentheses represent the proportion of the two sites determined from averaged inhibition curves of three to nine experiments conducted in duplicate. All other data represent the mean ± standard error of the mean of the stated number of experiments performed in duplicate.

	Rat reticulocyte			Rat erythrocyte			Rat lung K_i	
	n	K_i	n_H	*n*	K_i	n_H	*Beta*$_2$-component	*Beta*$_1$-component
		nM			*nM*		*nM*	*nM*
Beta$_1$-selective								
(±)-Atenolol	6	5,200 (±200)	1.03 ± 0.02	7	3,470 (±240)	0.97 ± 0.02	5,800 (80%)	40 (20%)
(±)-Practolol	6	37,000 (±3,400)	1.02 ± 0.01	7	27,400 (±1,000)	0.98 ± 0.02	46,500 (78%)	341 (22%)
(±)-Metoprolol	3	380 (±29)	0.97 ± 0.03	3	278 (±7)	0.94 ± 0.03	450 (83%)	21 (17%)
(±)-Betaxolol	3	202 (±12)	1.02 ± 0.01	0	—	—	201 (80%)	1.9 (20%)
Beta$_2$-selective								
(±)-Procaterol	8	26.2 (±1.8)	0.88 ± 0.02	6	32 (±2.7)	1.01 ± 0.04	41 (76%)	4,100 (24%)
(±)-ICI 118.551	5	1.10 (±0.04)	1.05 ± 0.03	6	0.96 (± 0.03)	1.03 ± 0.02	1.7 (83%)	107 (17%)

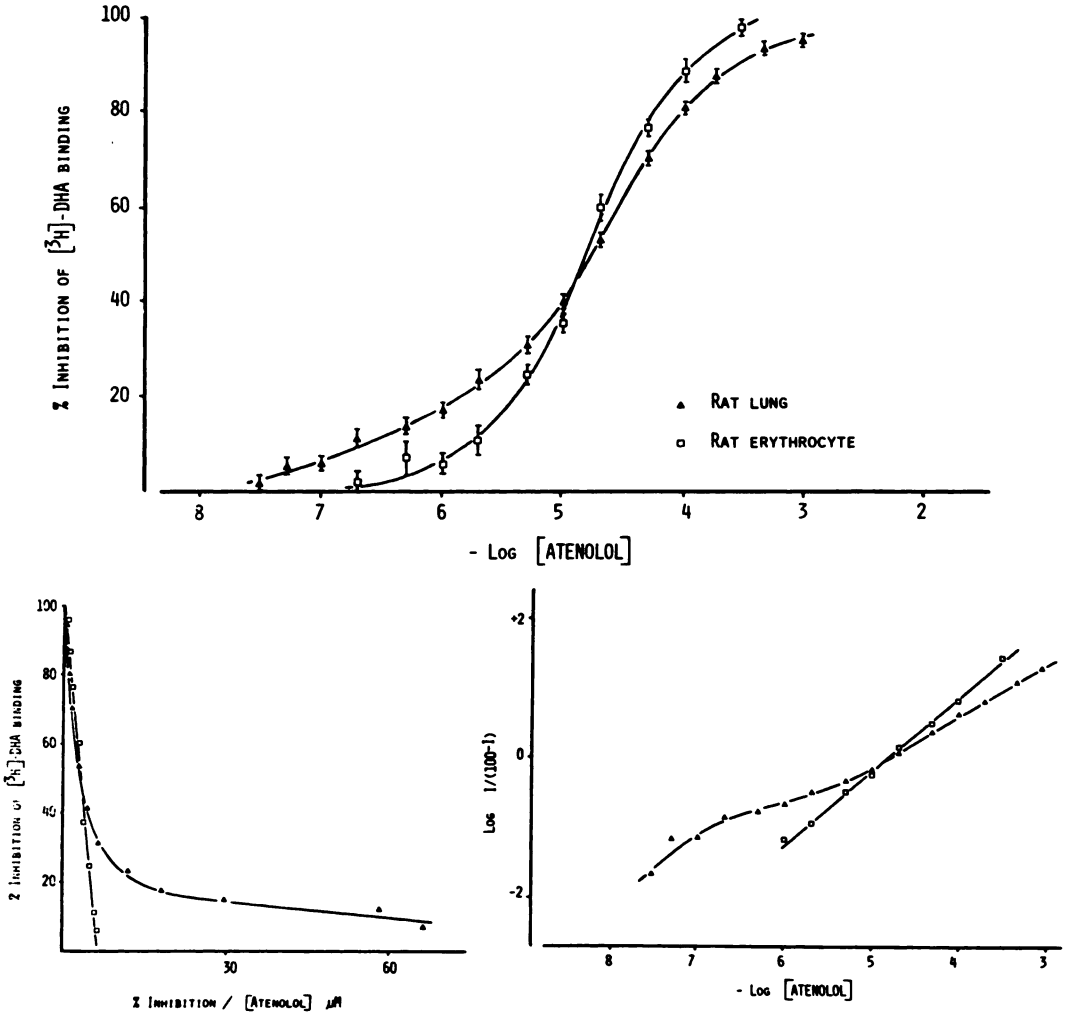

FIG. 4. *Inhibition of specific [^{3}H]DHA binding to rat lung (▲) and rat erythrocyte (□) membranes by (±)-atenolol (beta$_1$-selective)*
Drugs were incubated with the membranes in the presence of 1.5 mM [^{3}H]DHA and specific binding was assayed as described under Materials and Methods. The inhibition data have been analyzed by Hofstee plots (*I* versus *I*/[drug], where *I* is the percentage inhibition of specific [^{3}H]DHA binding) and by Hill plots (log *I*//(100−*I*) versus log[drug]. These data are the means ± standard error of the mean of nine (rat lung) and six (rat erythrocyte) experiments performed in duplicate.

ness of tissues to a series of natural and synthetic sympathomimetic agents (19). Supportive evidence for such a subdivision have confirmed that *beta*-receptors in heart and adipose tissue (*beta*$_1$) differ markedly in their affinity for some agonists and antagonists from those present in vascular, tracheal, and uterine smooth muscle (*beta*$_2$) (see ref. 20). However, because of the dissimilar pharmacological specificity of tissue preparations, such a strict subdivision of *beta*-adrenoceptors has been criticized as an oversimplification (21–23). Such findings have led to the suggestion that there may be more than two subtypes of *beta*-adrenoceptor (23, 24) or that both *beta*$_1$ and *beta*$_2$ receptors may coexist in the same organ (25–27) and that stimulation of both types of receptors could result in the same physiological response.

We have used radiolabeled ligand binding to overcome the inherent problems associated with estimation of drug-receptor interactions in intact pharmacological preparations (28). Our previous studies (1, 2, 11) were the first to present direct evidence that both *beta*-adrenoceptor subtypes could coexist in tissues, thereby providing support for the original hypothesis of Carlsson et al. (25). The present study has compared the binding of [^{3}H]DHA to membrane preparations of rat red blood cells with that to preparations of rat lung. This study was initiated in order to determine whether *beta*-adrenoceptor subtypes could coexist in a single homogeneous population of cells, and to characterize the *beta*-adrenoceptor present using recently synthesized, highly selective adrenergic agents. Of these, ICI 118.551 exhibited >100-fold *beta*$_2$-adrenoceptor selectivity using rat lung membranes, which was similar to that obtained with isolated pharmacological preparations (29); SL 75212 (betaxolol) showed 100-fold *beta*$_1$-adrenoceptor selectivity using rat lung membranes as compared with a "cardioselectivity" ratio of 220 using *in vitro* tissue preparations (30). Previous characterization of rat erythrocyte and reticulocyte *beta*-adrenoceptors have relied on the relative potency of agonists and the use of adrenergic antagonists with little selectivity (8, 10), and both *beta*$_1$ (10) and *beta*$_2$ (8, 9) adrenoceptor classifications have been suggested.

The results of our studies confirm the presence on rat

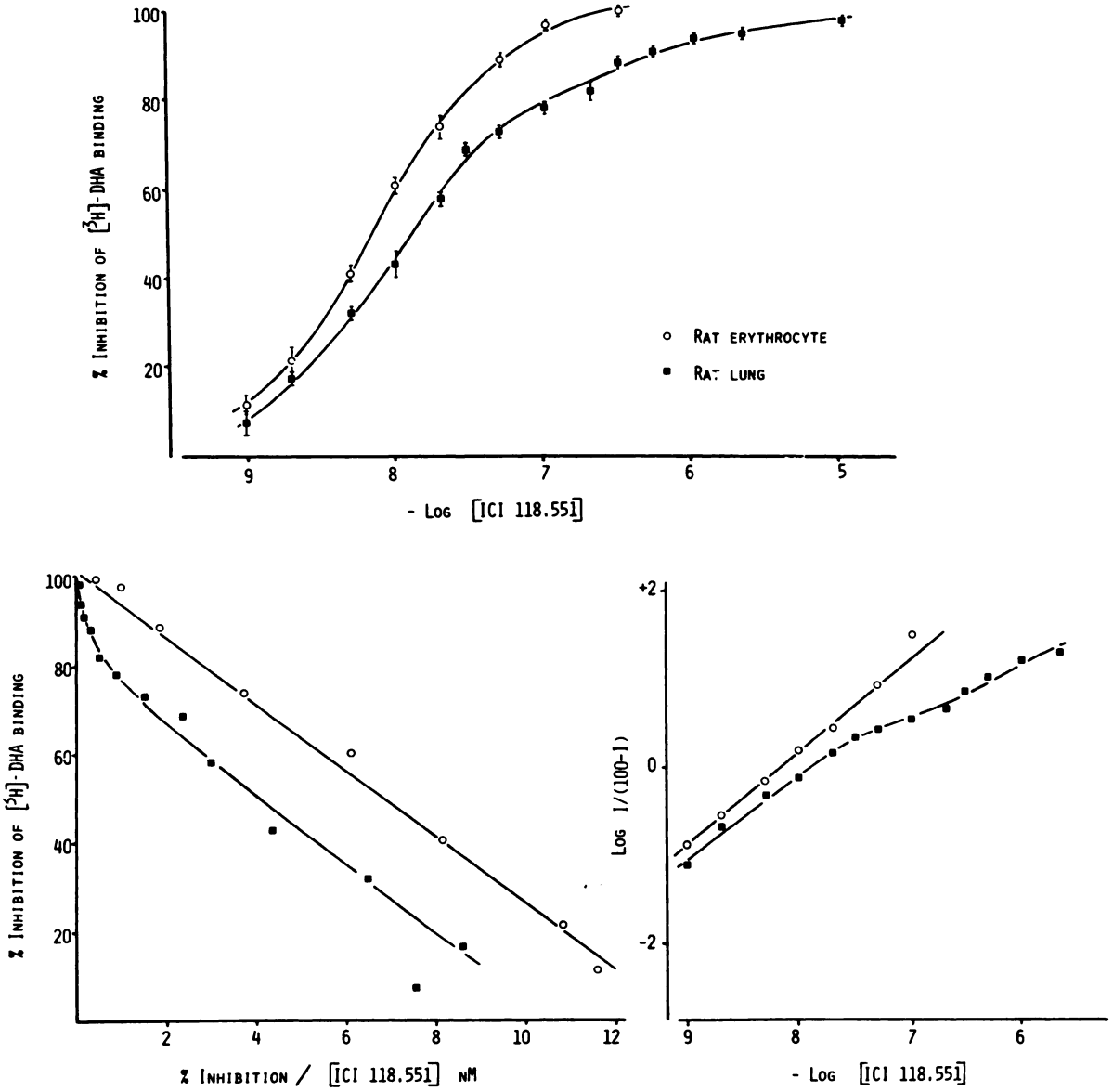

FIG. 5. *Inhibition of specific [^{3}H]DHA binding to rat lung (■) and rat erythrocyte (○) membranes by (±)-ICI 118.551 (beta$_2$-selective)* Experimental conditions were identical with those described in legend to Fig. 4. These data represent the means ± standard error of the mean of four (rat lung) and six (rat erythrocyte) determinations performed in duplicate.

erythrocytes and reticulocytes of [^{3}H]DHA binding sites which have all of the characteristics of *beta*-adrenoceptors. Thus, binding was stereoselective, saturable, of high affinity, rapid, reversible, and displaceable by drugs which interact at *beta*-adrenoceptors. The maximal number of binding sites for [^{3}H]DHA was increased in reticulocyte membranes by 290% as compared with erythrocytes, whereas the affinity of [^{3}H]DHA for these sites was unchanged. These findings were similar to those reported by others (5, 31, 32), differing only in the increase in the maximal number of binding sites. However, since receptor density is dependent on the degree of reticulocytosis (7), the higher values we obtained for maximal number of sites in reticulocytes was probably a reflection of the greater degree of reticulocytosis induced by our dosing regimen.

The characteristics of the *beta*-adrenoceptor binding sites on erythrocytes were, in almost all respects, identical with those present on reticulocytes. The association and dissociation rate contants and equilibrium dissociation constants for [^{3}H]DHA binding to both receptor preparations were not statistically different. In addition, the affinities of a series of agonists and antagonists in competition with [^{3}H]DHA binding to reticulocyte and erythrocyte *beta*-receptors were very similar, suggesting strongly that the receptors were identical. Similar conclusions have been reached by other workers using [^{125}I]hydroxybenzylpindolol (8, 9) and [^{3}H]DHA binding (31), and adenylate cyclase measurements (8).

The relative potencies of agonists displacing [^{3}H]DHA binding to rat reticulocytes and erythrocytes were identical with those displacing binding to rat lung membranes, and were indicative of *beta$_2$*-adrenoceptor classification as proposed by Lands *et al.* (19). Further sup-

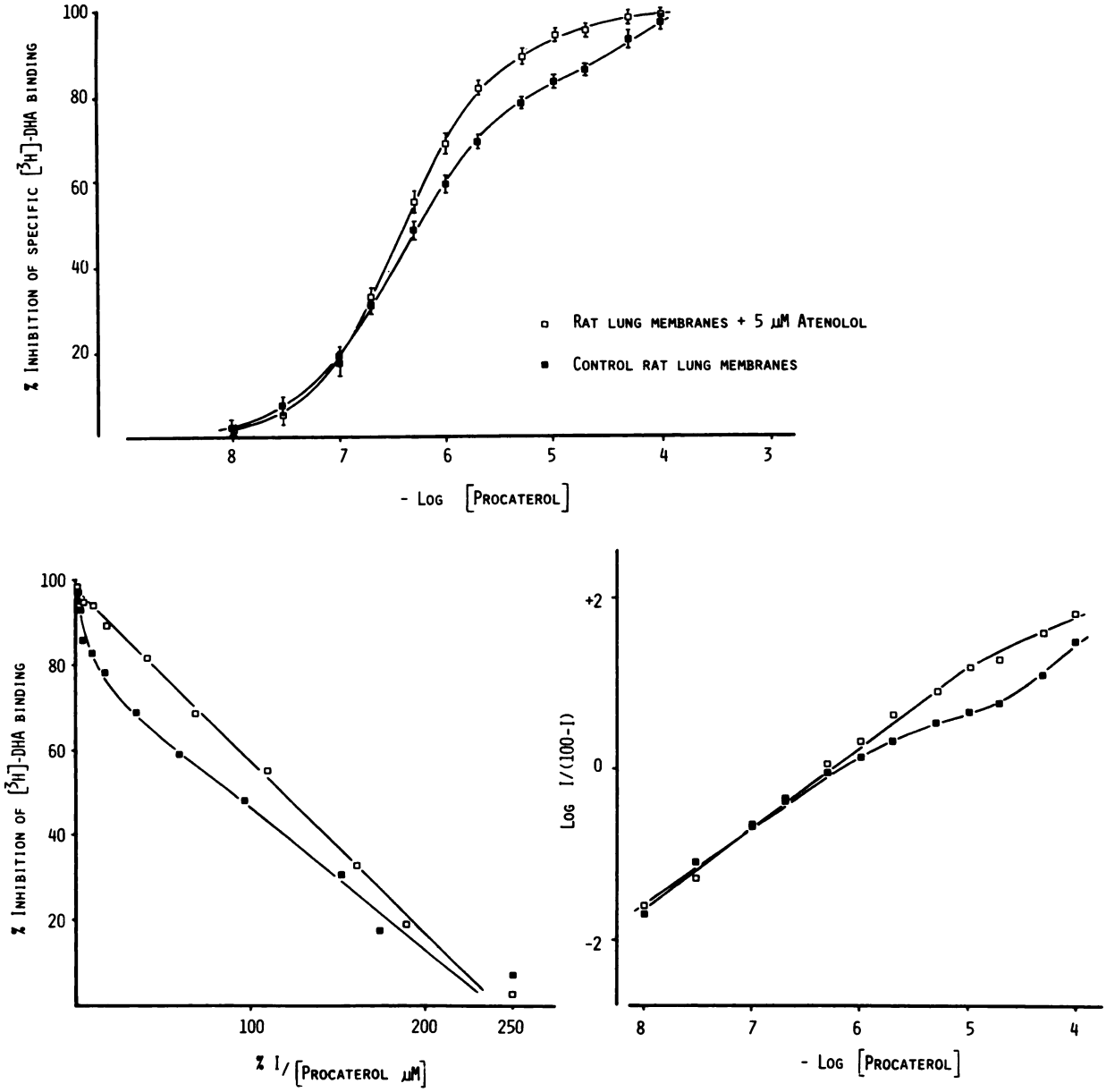

FIG. 6. *Inhibition of specific [^{3}H]DHA binding to rat lung membranes by (±)-procaterol in the absence (■) and presence (□) of 5μM atenolol*
Rat lung membranes were incubated for 15 min at 22° with (±)-atenolol or buffer and added to tubes containing [^{3}H]DHA (1.5 nM) and increasing concentrations of (±)-procaterol, yielding a final concentration of atenolol of 5 μM. After 30 min of incubation, the contents were diluted with 1 ml of ice-cold 50 mM Tris-HCl (pH 7.8) and filtered under reduced pressure. [^{3}H]DHA bound to the filters was determined as described under Materials and Methods. Data represent the mean ± standard error of the mean of four (control) and eight (atenolol-treated) experiments performed in duplicate.

port for this classification comes from our work with antagonists, which have been reported to show considerable *beta*$_1$ or *beta*$_2$ selectivity in intact preparations. The affinities of these drugs for rat erythrocyte and reticulocyte *beta*-adrenoceptors correlated well with their over-all affinities for the receptors of rat lung, which contain approximately 80% *beta*$_2$-adrenoceptors, and extremely well with their affinities at the *beta*$_2$-adrenoceptor binding site of rat lung membranes, when curves are dissected into *beta*$_1$ and *beta*$_2$ components. The displacement curves of selective agents in competition with [^{3}H] DHA binding to rat erythrocyte and reticulocyte membranes were indicative of a homogeneous receptor population with binding governed by the law of mass action. These data provide evidence that a single population of cells contains a homogeneous class of *beta*-adrenoceptors and that the heterogeneous nature of *beta*-receptors in lung tissue could reflect the presence of different cell types in that organ (33). However, in view of recent evidence suggesting that *beta*$_1$ and *beta*$_2$ adrenoceptors mediate catecholamine-induced bronchodilation, smooth muscle cells in lung may possess both receptor subtypes (27).

The present work confirms and extends previous observations on rat lung *beta*-adrenoceptors (1, 11). In these initial studies, displacement curves of selective agents

TABLE 3

IC_{50} values of drugs for $beta_1$ and $beta_2$ sites and proportions of each site

Analysis of antagonist inhibition curves of [^{3}H]DHA binding to rat lung membranes by the method of Hofstee (see ref. 1) and iterative computer-assisted curve-fitting of the raw data. The means of five to nine inhibition curves were analyzed by the two methods, as described in the text, and IC_{50} values (molar) and percentages of each site are indicated.

Drug	Hofstee analysis		Iterative curve-fitting	
	$Beta_2$ site	*$Beta_1$* site	*$Beta_2$* site	*$Beta_1$* site
(±)-ICI 118.551	7.1×10^{-9} 81%	4.9×10^{-7} 18%	8.5×10^{-9} 83%	5.3×10^{-7} 17%
(±)-Atenolol	3.1×10^{-5} 74%	2.4×10^{-7} 24%	3.1×10^{-5} 80%	2.1×10^{-7} 20%

were analyzed using Hofstee plots and indicated that both *$beta_1$* and *$beta_2$* adrenoceptors coexisted in membrane preparations in proportions of 1:3. The present study has utilized, in addition, a number of recently synthesized agents of high selectivity and has analyzed displacement data by iterative curve-fitting. We have confirmed the applicability of the previous data analysis for use with selective agents and have estimated the relative proportion of adrenoceptor subtypes as 80% *$beta_2$*, 20% *$beta_1$*. By using techniques similar to those described by us (1, 11) and employing iterative fitting of nonlinear Hofstee plots, Minneman *et al.* (12) have recently confirmed the presence in rat lung membranes of both *beta*-adrenoceptor subtypes in the ratio of approximately 85% *$beta_2$*, 15% *$beta_1$*.

Further evidence for the heterogeneous nature of *beta*-adrenoceptor binding sites on rat lung membranes has been provided by the demonstration in the present experiments that occupation of the *$beta_1$* sites with the selective agent atenolol results in a displacement curve for the *$beta_2$* agent procaterol that closely approximates to a single class of binding site which behaves according to the law of mass action. These results demonstrate that the minor binding site can be occupied by a *$beta_1$*-selective drug and result in a significant reduction in the degree of heterogeneity of the unoccupied beta-adrenoceptors in the membranes. This suggests that *$beta_1$* and *$beta_2$* adrenoceptors can coexist in tissues in a noninteracting manner and are probably quite separate entities. Present attempts to separate the receptors by solubilisation should provide more direct evidence for this concept.

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Send reprint requests to: Dr. S. R. Nahorski, Department of Pharmacology and Therapeutics, Medical Science Building, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom.