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β-ADRENERGIC RECEPTOR LIGAND BINDING BY RABBIT LUNG*

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Abstract—The β-adrenergic receptor antagonists, [^{14}C]propranolol and [^{3}H]dihydroalprenolol, and [^{3}H]dihydroergocryptine, an α-adrenergic receptor antagonist, were removed to the extent of 94, 84 and 78 per cent, respectively, from medium perfused through rabbit lung. Subcellular fractionation indicated that most (85–97 per cent) of the radiolabel was bound to particulate fractions of lung homogenates. Studies of [^{3}H]dihydroalprenolol binding to membrane preparations, *in vitro*, revealed the presence of high affinity ($K_D = 0.5$ nM) and high density (>1 pmole/mg of protein) β-adrenergic-specific binding sites. Binding was stereospecific since (−)-propranolol was 250-fold more potent than (+)-propranolol in inhibiting the binding of [^{3}H]dihydroalprenolol.

It is now clear that endothelial and other cells of the pulmonary vasculature are capable of metabolic functions which result in quantitative and qualitative modification of the pharmacological activity of many circulating vasoactive agents [1–3]. For example, the biogenic amines, 5-hydroxytryptamine and norepinephrine, are removed from pulmonary blood and metabolized by the lungs of many species [1, 4] including man [2]. The lung also removes and concentrates basic lipophilic amines [3, 5]; removal by lung of the β-adrenergic antagonist, propranolol, is particularly effective [6]. It was of interest, therefore, to compare the removal and the subsequent disposition of propranolol in the perfused lung with that of a more potent β-adrenergic antagonist, [^{3}H]dihydroalprenolol, and the α-adrenergic blocking agent, [^{3}H]dihydroergocryptine [7]. We were able to demonstrate a high degree of uptake of the three adrenergic receptor antagonists by isolated, perfused rabbit lung and relatively slow subsequent release. Furthermore, study of the binding of radioligand *in vitro* confirmed the presence of specific β-adrenergic ligand binding sites in membrane fractions of lung homogenates.

MATERIALS AND METHODS

Adult rabbits weighing 1.7 to 2.4 kg were anesthetized by i.v. injection of allobarbital (30 mg/kg) and urethane (120 mg/kg) after i.v. injection of 500 units heparin/kg. The thorax was opened by a midline sternotomy and the lungs were removed intact. The trachea and the right and left pulmonary arteries were cannulated. Simultaneous, independent perfusion of the lungs was performed with Krebs' medium (37°) at a rate of 10 ml/min, as described previously [8]. [^{14}C]Propranolol (PRO, 0.77 μM) perfusion was achieved by means of a single pass system [8] and fractions of the effluent from each lung were collected for 60- or 90-sec intervals. [^{3}H]Dihydroalprenolol (DHA, 10 nM) and [^{3}H]dihydroergocryptine (DHEC, 8.3 nM) were perfused through the lungs in a recirculating system in which the effluent from the lungs was returned to the reservoir containing perfusion medium and labeled drugs. After perfusion with radioactive drug for 10 min, the lungs were perfused with drug-free Krebs' medium (wash-out period) and fractions of the effluent were collected for 10 or 15 min. Aliquots of all fractions were added to 10 ml Aquasol (New England Nuclear Corp., Boston, MA), and the radioactivity was determined by means of a Packard Tri-Carb liquid scintillation spectrometer.

After wash-out, the lungs were removed from the perfusion apparatus, the large airways and vasculature were dissected away and the lungs were blotted and weighed. The lung tissue was then homogenized in 0.25 M sucrose containing 0.05 M *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.5, at 4° (referred to subsequently as "buffer") and separated by centrifugation into a supernatant and four particulate fractions by a modification of the method of Hook *et al.* [9], presented schematically in Fig. 1. Subcellular fractions isolated from lungs perfused with labeled drugs were dialyzed against 400 ml buffer at 0° for up to 22 hr. Aliquots were taken at various times and the retained radioactivity was analyzed by liquid scintillation counting.

Radioligand binding studies were carried out *in vitro* with aliquots (250 μg) of individual fractions which were added to 12 × 75 mm glass test tubes. The total reaction volume was 1.0 ml and contained 0.05 M HEPES, 0.25 M sucrose, and 5×10^{-4} M ascorbate at pH 7.5 and 37° unless otherwise stated. The reactants were preincubated for 5 min at 37° and the reaction was then initiated by the addition of [^{3}H]DHA or [^{3}H]DHEC. The reaction was terminated after 10 min

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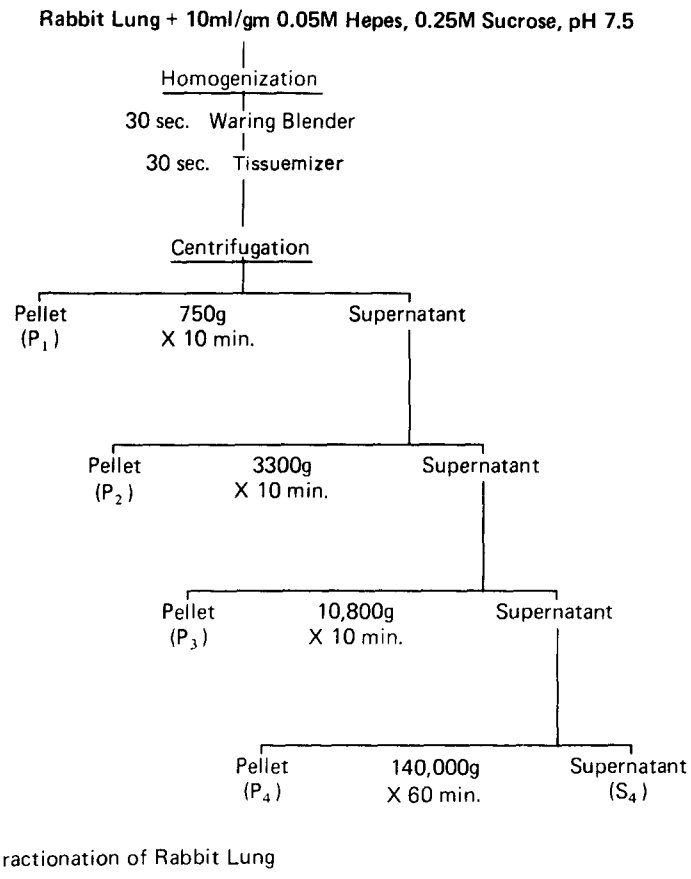

Fig. 1. Schematic diagram of the method of fractionation of lung as modified from Hook *et al.* [9]. The lung was homogenized in 0.05 M HEPES, 0.25 M sucrose, 10 ml/g, pH 7.5, at 4°.

by filtering 0.4-ml aliquots through Whatman GF/F filters which had been soaked overnight in 10 μM (±)-propranolol. Filters were then rinsed with 20 ml of ice-cold buffer. The filters were air dried, placed in scintillation counting vials, and then treated with 0.5 ml Protosol to digest protein adsorbed on the filters. The vials were capped tightly, heated to 55° and shaken for 30 min. After cooling the vials to room temperature, 10 ml Econofluor was added and the radioactivity in each was measured. Protein analyses were performed by the method of Lowry *et al.* [10] or by the Coomasie binding method [11], using bovine serum albumin as a standard.

[^{3}H]Dihydroalprenolol (48.6 Ci/m-mole), [^{3}H]dihydroergocryptine (24 Ci/m-mole), and [^{3}H]propranolol (21 Ci/m-mole) were obtained from the New England Nuclear Corp.; [^{14}C]propranolol was a gift from Ayerst, New York, NY. The purity of the radiolabeled compounds was verified by thin-layer chromatography. Catechol, (−)-isoproterenol, (−)-ascorbic acid, and (±)-propranolol were obtained from the Sigma Chemical Co., St. Louis, MO., HEPES from CalBiochem, La Jolla, CA., Econofluor and Protosol from New England Nuclear, and GF/F glass fiber filters from Whatman, Clifton, NJ.

Phentolamine and allobarbital were gifts from Ciba-Geigy, Summit, NJ. Dihydroergocryptine and (+)-propranolol were gifts from Sandoz, East Hanover, NJ, and (−)-propranolol from Ayerst.

RESULTS

[^{14}C]Propranolol, [^{3}H]dihydroalprenolol and [^{3}H]dihydroergocryptine were removed by perfused lung to the extent of 94, 84 and 78 per cent respectively. Junod [3] reported no intrapulmonary metabolism of propranolol after uptake. We confirmed this observation and also found that thin-layer chromatography of toluene extracts of perfused lung failed to reveal any evidence of metabolism of DHA or DHEC. The efflux of radioactivity after perfusion with these substances, which reflects unchanged ligand, is shown in Fig. 2. It can be seen that the loss of each ligand from the lungs is slow, although the rate of DHEC loss was more rapid than that of DHA or PRO.

In order to determine whether there was specificity of binding to different subcellular structures, the lungs were homogenized after perfusion and fractionated by differential centrifugation as described in Materials and Methods. According to the work of Hook *et al.* [9], these fractions (Fig. 1) correspond roughly to the following subcellular components; P_1-unbroken and partly disrupted cells and connective tissue; P_2-heavy mitochondria; P_3-light mitochondria; P_4-microsomes and S_4-soluble fraction. The distribution of label is shown in Table 1. Of the radioactivity remaining in the lung after perfusion, 97 per cent of [^{3}H]DHA was associated with particulate material; the corresponding figures for [^{14}C]PRO and [^{3}H]DHEC were 85 and 94

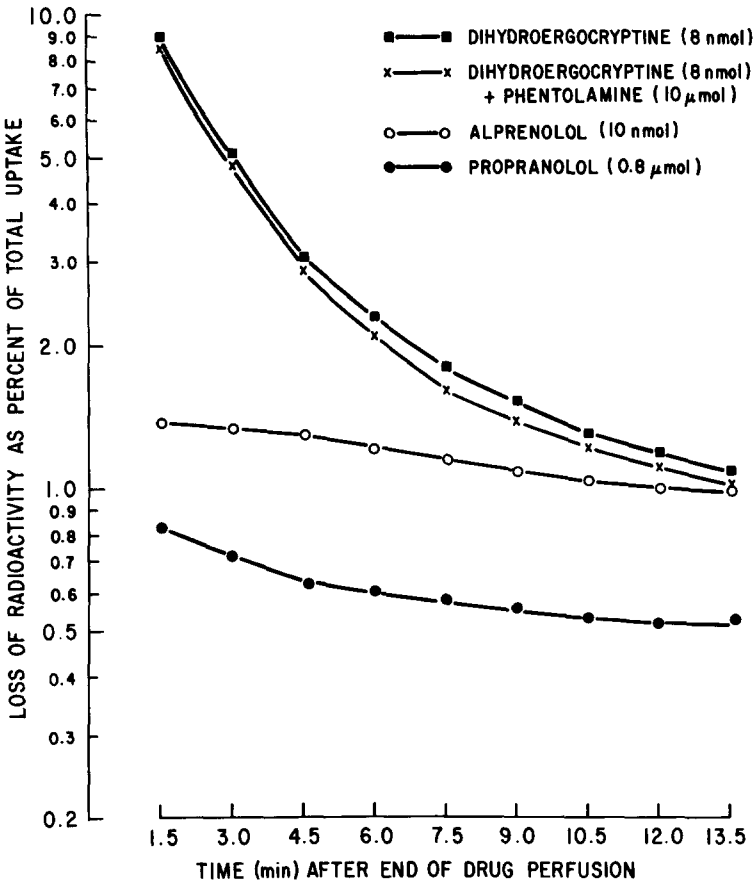

Fig. 2. Loss of adrenergic blocking agents from perfused lung. After perfusion with radiolabel, the perfusion medium was changed to Krebs' medium at 37° and perfusion was continued for another 10 min. Fractions were collected and analyzed for radiolabel. The abscissa indicates the time after initiation of washout and the ordinate indicates loss of radioactivity as per cent of total uptake.

per cent respectively. Most of the [^{14}C]PRO in the homogenates was associated with P_1, but the specific activity of labeling in P_2 and P_3 was 2–3 times higher than in other fractions. S_4 contained approximately 15 per cent of the total label, but its specific activity was 7-fold lower than that of all other fractions.

[^{3}H]Dihydroalprenolol perfusion resulted in a high degree of uptake. The percentage of label in fractions P_1, P_2 and P_3 was similar. Fraction P_1 contained approximately 15 per cent of the label, while S_4 contained only 3 per cent of the total label. The specific activity was similar in fractions P_2, P_3 and P_4 (178, 201 and 185 fmoles/mg of protein, respectively, in one experiment and 104, 118 and 112 in a second experiment). Fraction P_1 had 108 fmoles DHA/mg in one experiment and 69.2 fmoles/mg in a second experiment. The labeling of S_4 was about 10-fold lower than that of fractions P_2, P_3 and P_4. The distribution of [^{3}H]DHEC labeling was similar to that observed for [^{3}H]DHA.

Dialysis of labeled fractions was performed to determine whether the pattern of loss of label was distinct for any specific fraction. It can be seen (Fig. 3) that bound [^{14}C]PRO and bound [^{3}H]DHEC are lost from each fraction at approximately the same rate. However, loss

Table 1. Distributions of radioligands in subcellular fractions*

Radiolabel	n	Fraction P_1 (%)	P_2 (%)	P_3 (%)	P_4 (%)	S_4 (%)
[^{3}H]DHA	2	15.5	23.5	26.9	25.0	3.1
[^{3}H]DHEC	5	17.4 ± 1.47	31.0 ± 2.37	26.4 ± 0.748	18.8 ± 1.56	6.40 ± 0.311
[^{14}C]Propranolol	3	43.4 ± 2.63	13.0 ± 1.15	16.3 ± 0.882	11.8 ± 1.36	14.7 ± 0.333

*The various fractions were prepared from lungs perfused with each drug as described under Materials and Methods. Results presented are the means of n determinations ± S. E.

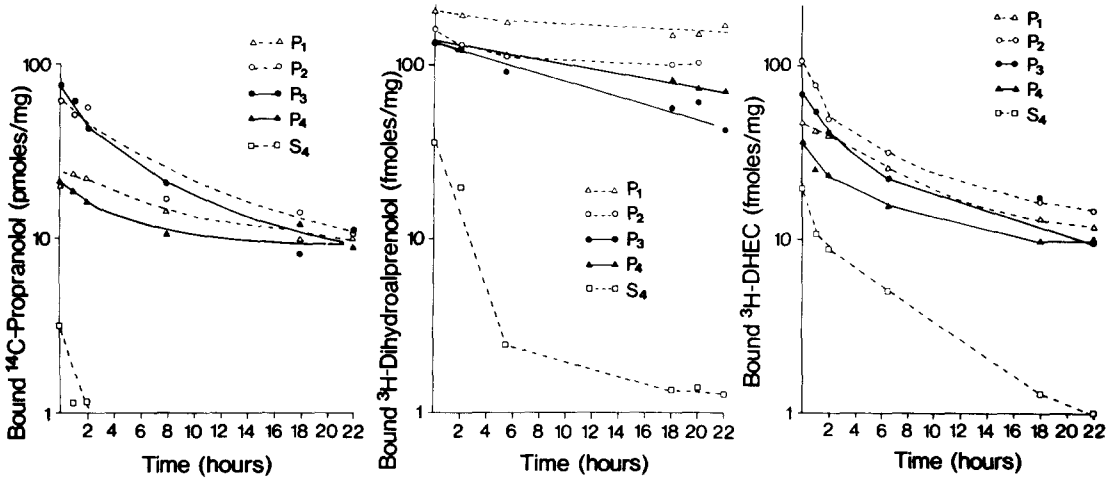

Fig. 3. Loss of radioactivity on dialysis of fractions prepared from lung perfused with 0.77 μM [^{14}C]propranolol, 8.3 mM [^{3}H]DHEC or 10 nM [^{3}H]DHA. The dialyzing medium was 0.05 M HEPES, 0.25 M sucrose, at 4° and pH 7.5. Aliquots were removed from dialysis at various times and added to 10 ml Aquasol; then radioactivity was determined.

of [^{3}H]DHA from all particulate fractions is slower and the curves approximate straight lines (i.e. single exponential processes) more closely than was observed for loss of either [^{14}C]PRO or [^{3}H]DHEC. The rate of loss of the three ligands from S_4 was more rapid than from any other fraction.

In order to examine the possibility that uptake of these adrenergic antagonists by the lungs was related to their adrenergic blocking properties, binding studies were carried out on isolated fractions of lung homogenates prepared as described in Materials and Methods. Binding of 1 μM propranolol was studied, but because

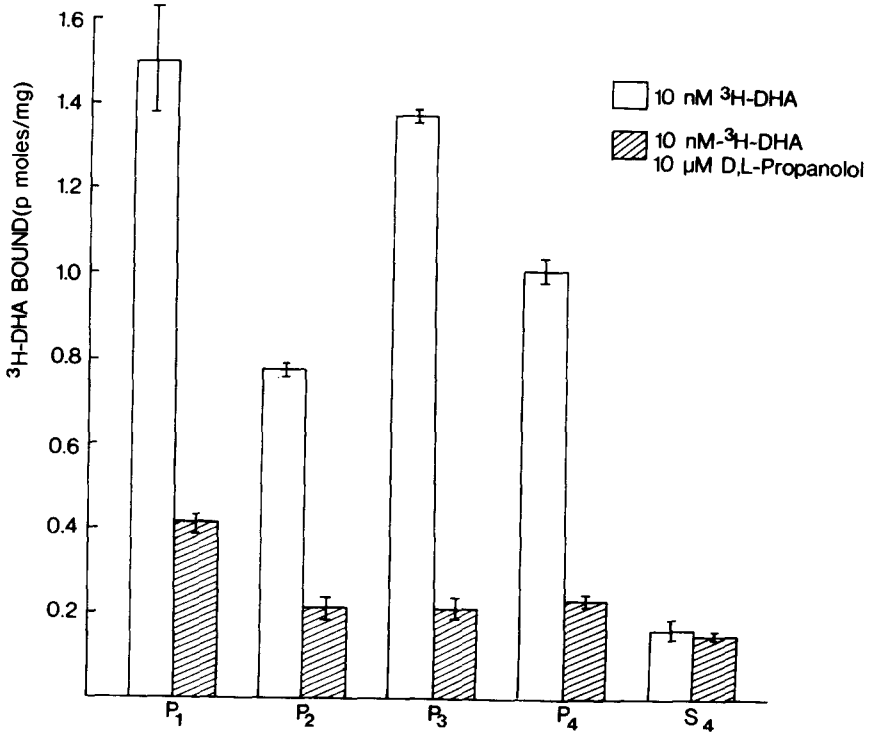

Fig. 4. Binding *in vitro* of [^{3}H]DHA (10 nM) to subcellular fractions prepared from lung homogenates. The open bars indicate total binding and the crosshatched bars binding of [^{3}H]DHA in the presence of 10 μM (±)-propranolol. The difference between binding in the presence and absence of propranolol is taken to reflect β-adrenergic receptor labeling. The results presented are the means of quadruplicate determinations ± S. E.

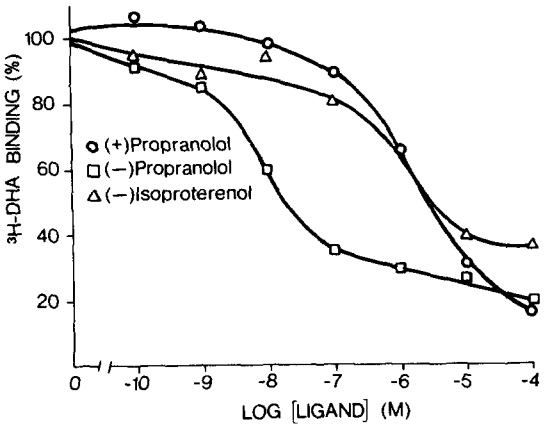

Fig. 5. Competitive inhibition of binding of [^{3}H]DHA (10 nM) to fraction P_1 by (−)-propranolol, (+)-propranolol and (−)-isoproterenol. The abscissa indicates the concentration of competing ligand and the ordinate the per cent of total binding. The results are the means of quadruplicate determinations.

of a high degree of non-specific binding to the filters and denatured protein no clear conclusions could be drawn. [^{3}H]dihydroalprenolol binding to individual fractions is shown in Fig. 4. Particulate fractions were labeled by [^{3}H]DHA to the extent of approximately 1 pmole/mg of protein. This binding exceeds by 10-fold that seen in crude membrane preparations from other tissues [11]. It can be seen (Fig. 4) that, while all fractions bind [^{3}H]DHA, incubation in the presence of 10 μM (±)-propranolol greatly reduces the amount bound. Thus, specific binding of DHA, defined as the total minus "non-specific" binding, is evident.

Figure 5 demonstrates inhibition of [^{3}H]DHA binding to P_1 by various concentrations of (−)-isoproterenol and (−)- and (+)-propranolol. (−)-Isoproterenol (0.5 μM) inhibits 50 per cent of the [^{3}H]DHA binding at a concentration of about 1.0 μM, while the corresponding concentration for (+)-propranolol is approximately 1.5 μM. The more potent stereoisomer, (−)-propranolol, inhibits 50 per cent of the binding at a concentration of 6 nM; this ratio of potency of (+)/(−)-propranolol is approximately 250. If we assume that (−)-isoproterenol, (−)-propranolol and (+)-propranolol act competitively to inhibit the binding of [^{3}H]DHA, then we can apply the formula given by Cheng and Prusoff [13] to determine the inhibition constant (K_I) for each drug. The K_I values thus determined are: 27 nM for (−)-isoproterenol, 75 nM for (+)-propranolol and 0.3 nM for (−)-propranolol.

The concentration dependence of [^{3}H]DHA binding to P_1 is demonstrated in Fig. 6. The difference between total binding (top curve) and that measured in the presence of 10 μM (±)-propranolol indicates binding specific for β-receptors. The curve for total binding shows an inflection point between 20 and 30 mM [^{3}H]DHA. These data may reflect the fact that binding to P_1 is heterogeneous and that binding at low concentrations of [^{3}H]DHA to other, non-specific sites, from which it may also be displaced by (+)-propranolol, occurs at higher ligand concentrations.

Figure 7 shows the concentration dependence of [^{3}H]DHA binding to fraction P_3 (light mitochondria). This curve shows no inflection point, which might be expected in that P_3 is a less heterogeneous fraction than P_1. From the magnitude of [^{3}H]DHA binding in the presence and absence of 10 μM (±)-propranolol (Fig. 7) we can estimate that the [^{3}H]DHA binding to P_3 is 50% saturated at 0.5 nM. This K_D value is in good

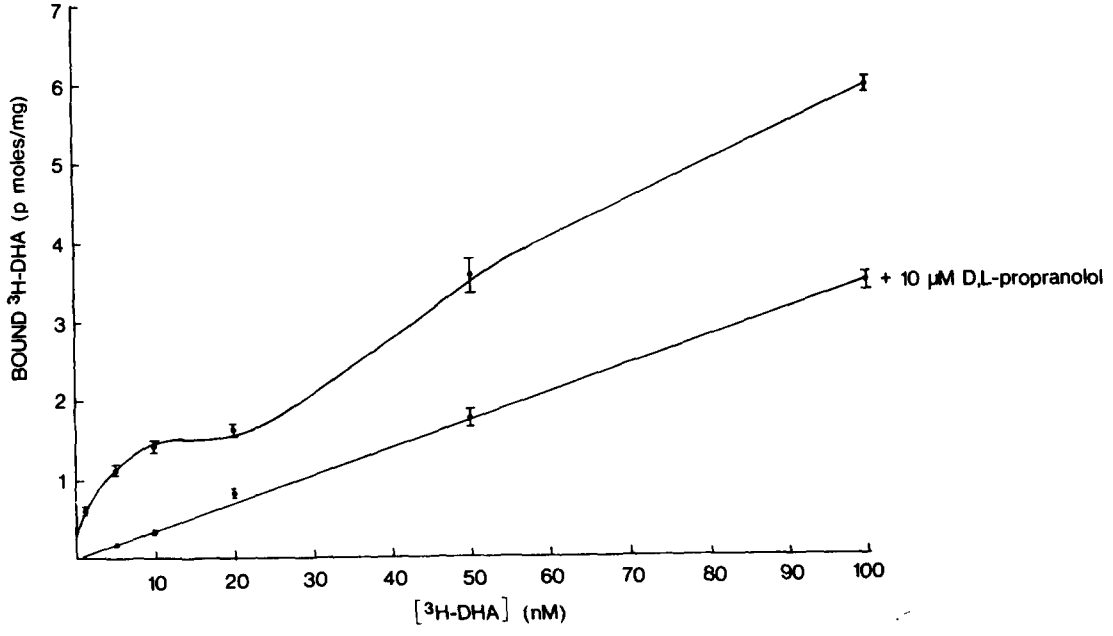

Fig. 6. Binding of [^{3}H]DHA to fraction P_1 at 37°, pH 7.5, as a function of the concentration of [^{3}H]DHA. P_1 was incubated for 10 min with varying concentrations of [^{3}H]DHA at a P_1 concentration of 0.25 mg protein/ml. Binding was determined by the filter assay described in Materials and Methods. The results are the means of quadruplicate determinations ± S. E.

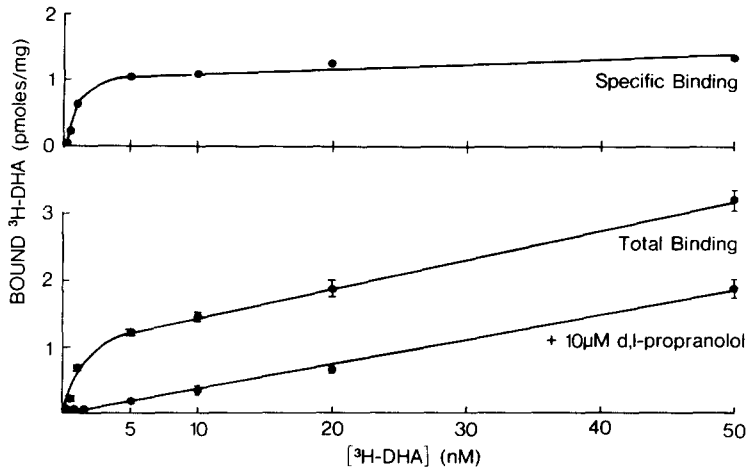

Fig. 7. Binding of [^{3}H]DHA to fraction P_3 as a function of the concentration of [^{3}H]DHA. Conditions were the same as in Fig. 6. The results presented are the means of quadruplicate determinations ± S. E.

agreement with values of 2 nM and 0.6 nM determined in equilibrium and kinetic studies, respectively, of the soluble β-adrenergic receptor in frog erythrocytes [12].

DISCUSSION

The central location in the circulatory system of the lungs allows them to modify the concentrations of drugs and vasoactive hormones in the cardiac output. We observed that in a single pass through the lungs 94 per cent of the perfused propranolol was removed, confirming previous reports of extensive uptake of this β-adrenergic blocking agent [6]. Our observation is clinically significant in that the concentration perfused (0.77 μM) is within the range of blood concentrations following intravenous administration of propranolol to patients [14]. Any manipulations which affect this uptake, or cause subsequent release of propranolol, therefore, may have serious pharmacological consequences to the patient, since there could be a sudden change in the circulating blood level of this drug.

Our studies have also demonstrated that 84 per cent of the [^{3}H]DHA and 78 per cent of the [^{3}H]DHEC perfused through the lung was retained. In an attempt to define specific subcellular binding sites, we determined the distribution of each antagonist among the different subcellular fractions isolated. The separation of organelles from lung homogenates is not as well defined as for the liver [9] because the former organ is composed of about 40 cell types [15]. Furthermore, elastin and collagen, which are major components of the lungs, are resistant to homogenization and separation by standard techniques [9]. Also, there is no assurance that organelles and β-receptor binding sites from different cell types will be homogeneous. Accordingly, the absence of discrete subcellular labeling may be related to the heterogeneity of each fraction (Table 1). One obvious conclusion from our data is that the drugs are bound relatively little to the soluble proteins which constitute approximately one-half of the total protein in homogenates. Fraction P_1 contained about 43 per cent of the perfused [^{14}C]propranolol, whereas only 17 per cent of the total [^{3}H]DHEC of [^{3}H]DHA in homogenates was associated with P_1 (Table 1). The high degree of [^{14}C]propranolol binding to P_1 reflects the extensive non-specific binding which makes this β-receptor specific ligand difficult to use in these studies [16].

Dialysis of labeled fractions (Fig. 3) reveals that fraction S_4, containing soluble proteins, loses radioactivity rapidly. Dialysis of [^{3}H]DHEC and [^{14}C]PRO labeled fractions demonstrated strikingly similar curves of loss of label with the respective drugs. Furthermore, the curves of loss from fractions P_1, P_2, P_3 and P_4 suggest that both [^{14}C]PRO and [^{3}H]DHEC were bound at multiple sites with differing dissociation constants. The rate of loss of [^{3}H]DHA on dialysis of radiolabeled fractions is slower than that of the other two drugs. In this case (Fig. 3), the loss of label by fractions P_1, P_2, P_3 and P_4 can be approximated by straight lines, suggesting that [^{3}H]DHA is bound predominantly to one type of binding site with a single dissociation constant. The calculated dissociation constant for P_3 is 1.00×10^{-3} min^{-1} which is of the same order of magnitude as that reported for the soluble β-receptor from frog erythrocytes [12].

Binding studies performed *in vitro* on isolated subcellular fractions of lung fractions indicate: (1) that non-specific binding of [^{3}H]propranolol resulted in data which were inconclusive; and (2) that [^{3}H]DHA uptake and binding in the lung are similar to those described in frog erythrocyte membrane [12].

The K_D for [^{3}H]DHA is 0.5 nM which is consistent with data obtained with other systems [12, 17]. The amount of specific binding, approximately 1.2 pmoles/mg at 10 nM [^{3}H]DHA, is 10-fold higher than in crude preparations of frog erythrocytes but is about the same as that reported for purified frog erythrocyte membranes. Our data show further that (−)-isoproterenol competes with [^{3}H]DHA for binding sites and that the

concentration of (−)-isoproterenol at which half the binding is inhibited is 0.54 μM. The 250-fold greater potency of (−)- than (+)-propranolol is strong evidence that the sites specifically binding [^{3}H]DHA are β-receptors.

Our data suggest that the lung may function as a "reservoir" for these potent adrenergic blocking drugs and, therefore, may greatly alter circulating concentrations of β-adrenergic antagonists and agonists. Considering the large surface area of the lung and the apparently high density of β-receptors throughout lung membranes (Fig. 4; P_1, P_2 etc.), it is possible that β-adrenergic receptors, although classically associated with airway muscle, are also present in vascular smooth muscle. Attempts are now underway to define β-receptors in pulmonary vasculature, which may be functionally accessible from the pulmonary circulation.

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REFERENCES

1. C. N. Gillis and J. A. Roth, *Biochem. Pharmac.* **25**, 2547 (1976).
2. C. N. Gillis and N. M. Greene, in *Metabolic Functions of the Lung* (Eds. Y. S. Bakhle and J. R. Vane), p. 173. Marcel Dekker, New York (1977).
3. A. F. Junod, in *Lung Metabolism* (Eds. A. F. Junod and R. Haller), p. 219. Academic Press, New York (1975).
4. A. P. Fishman and G. G. Pietra, *New Engl. J. Med.* **291**, 953 (1974).
5. R. M. Philpoxt, M. W. Anderson and T. S. Eling, in *Metabolic Functions of the Lung* (Eds. Y. S. Bakhle and J. R. Vane), p. 123. Marcel Dekker, New York (1977).
6. C. T. Dollery and A. F. Junod, *Br. J. Pharmac.* **57**, 67 (1976).
7. L. T. Williams, D. Mullikin and R. J. Lefkowitz, *J. biol. Chem.* **251**, 6915 (1976).
8. C. N. Gillis and Y. Iwasawa, *J. appl. Physiol.* **33**, 404 (1972).
9. G. E. R. Hook, J. R. Bend, D. Hoel, J. R. Fouts and T. E. Gram, *J. Pharmac. exp. Ther.* **182**, 474 (1972).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. M. M. Bradford, *Analyt. Biochem.* **72**, 243 (1976).
12. M. G. Caron and R. J. Lefkowitz, *J. biol. Chem.* **251**, 2374 (1976).
13. Y. Cheng and W. H. Prusoff, *Biochem. Pharmac.* **22**, 2099 (1973).
14. D. G. McDevitt and D. S. Shand, *Clin. Pharmac. Ther.* **18**, 708 (1975).
15. C. Kuhn III, in *The Biochemical Basis of Pulmonary Function* (Ed. R. Crystal), p. 3. Marcel Dekker, New York (1977).
16. D. E. Vatner and R. J. Lefkowitz, *Molec. Pharmac.* **10**, 450 (1974).
17. M. E. Maguire, E. M. Ross and A. G. Gilman, in *Advances in Cyclic Nucleotide Research* (Eds. P. Greengard and G. A. Robison), Vol. 8, p. 1. Raven Press, New York (1977).