Lung (1985) 163:161–171

Lung
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In Vitro Studies on the Relative Potency of Bronchodilator Agents

Joseph H. Fleisher and Jacob L. Pinnas*

Department of Internal Medicine, Arizona Health Sciences Center, Tucson, Arizona 85724, USA

Abstract. This study describes a rapid in vitro assay for the order of potency of bronchodilator drugs using specific binding of (−)-[^{3}H] dihydroalprenolol ([^{3}H]DHA) to rat lung membranes. Under linear conditions with respect to tissue, specific binding of [^{3}H]DHA showed saturability, rapid kinetics of association and dissociation of radioligand, and sterospecificity. Nanomolar (nM) concentrations for 50% inhibition (IC_{50} ± SE) for the bronchodilator drugs examined were as follows: albuterol, 1485 ± 170; isoproterenol, 136 ± 53; procaterol, 162 ± 28; terbutaline, 3310 ± 934; and zinterol, 51 ± 8.3. A comparison of binding studies using rat lung tissue membranes and similar preparations of rat heart and skeletal muscle demonstrated that lung tissue had 7 to 8 times more receptor sites (B_{max}) for [^{3}H]DHA than heart or skeletal muscle. Adenyl cyclase activity of the rat lung membrane preparation almost doubled in the presence of (−)-isoproterenol. Displacement of specific (^{3}H)DHA binding in membrane preparations may provide useful data for evaluating bronchodilator compounds.

Key words: [^{3}H] Dihydroalprenolol—Beta-adrenergic bronchodilator compounds—Rat lung membranes—Adenylate cyclase activity—Cyclic adenosine monophosphate (cAMP).

Introduction

Adrenergic responses elicited by exogenous catecholamines are designated as "beta" when their administration causes positive inotropic and chronotropic cardiac responses or smooth muscle relaxation [17]. Lands et al. [8] subdivided

* To whom offprint requests should be sent at Department of Internal Medicine, Arizona Health Sciences Center, Tucson, Arizona 85724, USA

beta-adrenergic responses into $beta_1$ effects, including those on cardiac muscle, and $beta_2$ effects, which include bronchodilation and uterine relaxation. Beta-adrenergic responses are antagonized by synthetic compounds such as propranolol and dihydroalprenolol. The latter compound labeled with tritium has been used for biochemical studies in vitro of beta-adrenergic receptor binding sites [18]. More recently, (−)-[^{3}H] dihydroalprenolol ([^{3}H]DHA) was used by Barnes et al. [1] to study radioligand binding to human lung adrenoceptors. They reported that butoxamine, a selective antagonist of $beta_2$-adrenergic responses [11], was approximately 100 times more potent than atenolol, a $beta_1$-antagonist [6], in displacement of [^{3}H]DHA binding indicating that the population of human lung beta-adrenoceptors is predominantly of the $beta_2$ subtype. Rugg et al. [16] reported a similar finding in rat lung tissue which showed 75% $beta_2$-adrenoceptor sites, compared with rabbit lung which contained only 40% $beta_2$-adrenoceptor sites. Because rat lung contains predominantly $beta_2$-adrenoceptors, we chose rat lung as a source for a membrane preparation to study the relative potency of bronchodilator drugs. The first set of experiments presented in this study quantitates [^{3}H]DHA binding sites in rat lung, heart, and skeletal muscle membrane preparations. Rat lung membranes were then used to study the kinetics, reversibility, and stereospecificity of [^{3}H]DHA binding. Several bronchodilator drugs were examined for their capacity to inhibit the specific binding of [^{3}H]DHA to the rat lung preparation.

Inasmuch as beta-adrenergic effects are mediated by stimulation of the enzyme adenylate cyclase [10], the rat lung membrane preparation was tested for its adenylate cyclase activity in the absence and presence of 1 μM (−)-isoproterenol.

Materials and Methods

Tritium-labeled (−)-DHA (47.4 Ci/mmol) in absolute ethanol was purchased from New England Nuclear, Boston, Mass. and stored at −20°C. Appropriate dilutions in water were made daily for each study. The following drugs were received as gifts: (−)-isoproterenol bitartrate from Sterling Winthrop; (−)-zinterol hydrochloride from Mead Johnson; (−)-procaterol hydrochloride from Warner Lambert; albuterol (salbutamol) from Schering; terbutaline sulfate from Ciba-Geigy; (−)-propranolol and (+)-propranolol from Ayerst Laboratories.

Tissue Preparation

Male Sprague-Dawley rats (225–300 g) were sacrificed by decapitation and the lungs and heart rapidly perfused with cold 0.9% NaCl. The organs were quickly excised, trimmed of connective tissue, large blood vessels, and fat, then blotted and weighted. The organs were minced with scissors and homogenized with 9 volumes of ice-cold 0.05 M Tris-HCl buffer containing 1 mM $MgCl_2$ (pH 8.0 at 25°C). Tissue was homogenized with three 10-sec bursts with a Brinkman Polytron at number 5 setting separated by 30-sec. pauses. The tissue homogenates were filtered through 4 layers of gauze, then centrifuged at 500 g (4°C) for 10 min to remove undisrupted cells and nuclei. The supernatant was then centrifuged at 40,000 *g* (4°C) for 15 min. The pellet was resuspended in the same buffer and centrifuged at 40,000 *g* again. This final pellet was suspended in the 50 mM Tris-HCl, 1 mM $MgCl_2$ buffer (pH 8.0) to a concentration approximately 1–2 mg protein/ml.

Radioligand Binding Assay

The incubation medium was that used for tissue preparation above. Specific [^{3}H]DHA binding was determined experimentally from the difference between counts bound in the absence (total binding) and presence of 1 μM (−)-propranolol (nonspecific binding). Routinely, 100 μl of tissue suspension was added to the [^{3}H]DHA in a final volume of 2.0 ml of incubation medium. After 1 hr of reaction time, (25°C) each incubation mixture was filtered through a GF/B glass fiber filter (Whatman) under reduced pressure. The filters were rapidly rinsed 3 times with 5 ml ice-cold buffer. The [^{3}H]DHA retained upon the filter was extracted overnight with 8 ml of scintillation fluid (2 L toluene, 1 L Triton X-100, and 16 g Omnifluor). Quantitation was by liquid scintillation spectroscopy with an efficiency of 45%. All experimental values were determined in duplicate. Conditions used limited total binding to less than 10% of the radioactivity in the medium so that the concentration of the radioligand did not change appreciably during the assay.

Adenyl Cyclase Activity

The assay was carried out as described by Chen et al. [4]. The lungs were prepared as for the [^{3}H]DHA binding studies, but the tissue was homogenized in a buffer consisting of 0.25 M sucrose, 10 mM Tris-HCl at pH 7.5, 1 mM $MgCl_2$, and 0.1 mM EDTA. Twenty μl of the tissue suspension was added to 160 μl of reaction buffer containing 20 mM Tris-HCl at pH 7.5, 8 mM theophylline, 10 mM creatine phosphate, 10 mM $MgCl_2$, and 14.5 μg of creative phosphokinase. The mixture was preincubated at 37°C for 5 min before the reaction was started by the addition of 20 μl of ATP and GTP (final concentration of 1.3 mM and 0.01 mM, respectively). After 10 min at 37°C, the reaction was stopped by adding EDTA to 3 mM (final concentration) and placing in a boiling water bath for 7 min. The mixture was centrifuged in a microfuge (Beckman) for 2 min and the supernatant removed for measurement of cAMP.

Determination of cAMP was based on a modification of the technique of Brown et al. [3] using an assay kit from Amersham-Searle Corporation. Fifty μl of the supernatant or diluted supernatant from the adenylate cyclase incubation was added to 150 μl of mixture consisting of 50 mM Tris-HCl (pH 7.5); 4 mM EDTA, binding protein for cAMP, and [8-^{3}H] cAMP. After incubation in a ice bath for 2 hr, 100 μl of activated charcoal suspension was added to absorb the free cAMP. The samples were centrifuged at 10,000 *g* 1 min. One-hundred μl of supernatant was transfered to vials containing 10 ml of scintillation fluid. Samples were counted in a liquid scintillation counter with 45% efficiency. In each experiment, a standard cAMP curve was constructed (range, 1–16 pmol/50 μl) and the cAMP level of the unknown sample was read from the standard curve.

Protein concentration of the tissue suspensions in the above studies were determined by the method of Lowry et al. [12].

Results

Tissue Linearity

Specific [^{3}H]DHA binding increases linearly with increasing tissue concentration up to 0.3 mg/ml of membrane protein for lung and skeletal muscle and 0.2 mg/ml for heart tissue membranes under the condition of assay (Fig. 1).

Saturation of [3H]DHA Binding to Rat Lung, Heart, and Skeletal Muscle Membranes

The saturability of specific [^{3}H]DHA binding to membranes prepared at 40,000 *g* from the above rat tissues was measured as a function of the added [^{3}H]DHA

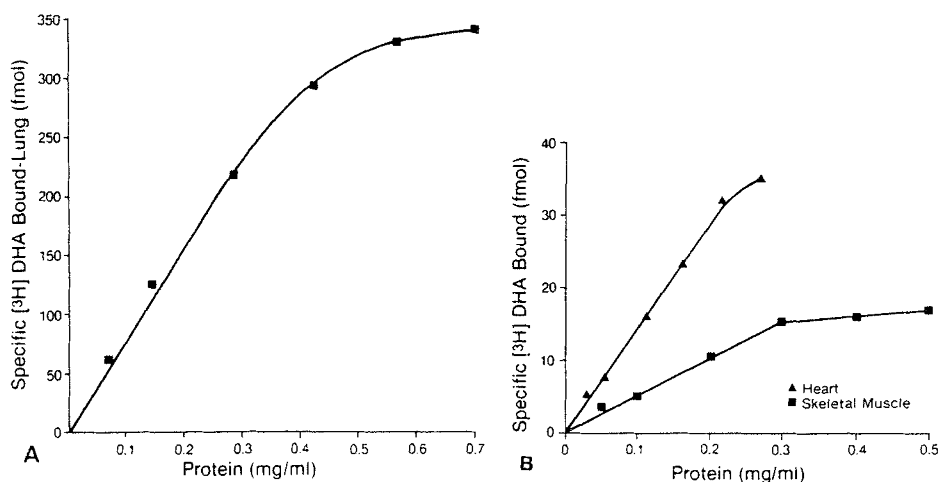

Fig. 1A, B. Effect of tissue concentration on specific [^{3}H]DHA binding to rat lung, heart, and muscle 40,000 g membranes. Specific [^{3}H]DHA binding was determined as described under "Methods." Total [^{3}H]DHA concentration was 0.53 nM. Total volume = 2 ml; temperature = 25°C; pH = 8.0. Incubation time was 60 min. Incubations were started by the addition of tissue. The curve shows the mean value for three trials with each point determined in duplicate. SEM less than ± 10%.

concentration. For each tissue the saturation isotherm resembled a rectangular hyperbola, which suggested that the membrane suspension contained a population of high-affinity beta-adrenergic receptors. Scatchard analysis of the saturation curves permitted the calculation of the dissociation constant, K_D and an estimate of the number of specific binding sites for [^{3}H]DHA, designated B_{max}, in the membranes derived from each tissue. Figure 2 shows the saturability of specific binding for [^{3}H]DHA and Scatchard analysis of this saturation curve for rat lung membranes. Similar saturation curves were obtained for specific binding of [^{3}H]DHA to rat heart and skeletal muscle membranes (not shown). The values for K_D and B_{max} derived from Scatchard plots are listed in Table 1.

Kinetics of [^{3}H]DHA Binding to Rat Lung Tissue

At 25°C the association of [^{3}H]DHA with rat lung receptor reached equilibrium by about 20 min (Fig. 3A). Bound radioligand was assayed at various time intervals up to the steady level (B_{eq}). The

$$L_n \frac{B_{eq}}{B_{eq} - B_t}$$

was plotted vs. time where B_t is the amount of specifically bound ligand at time, t (Fig. 3B). The slope of this line (K_{obs}) is related to the association (k_1) and dissociation (k_{-1}) rate constants and free ligand concentration [L] as follows:

Table 1. Apparent dissociation constants, K_D, and number of receptor sites (B_{max}) for [³H]DHA in rat lung, heart, and skeletal muscle

Tissue	K_Da (nM)	B_{max}a (fmol/mg protein)
Lung	0.26	680
Heart	0.32	96
Skeletal muscle	0.57	83

[a] The values shown are the means derived from Scatchard plots of the data from four experiments performed in duplicate with increasing concentrations of [³H]DHA. SEM less than ± 10%.

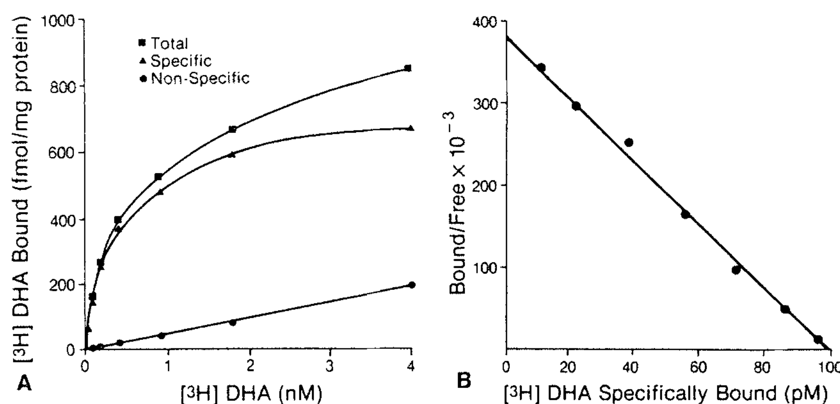

Fig. 2A [³H]DHA binding to rat lung membranes as a function of increasing concentrations of [³H]DHA. A direct plot of the data showing total binding, nonspecific binding in the presence of 1 μM (−)-propranolol, and specific binding by the difference between total and nonspecific binding. The points shown are the means of four trials conducted in duplicate. SEM less than ± 10%. **B** Scatchard plot derived from the data shown in **A**. Mean values for the correlation coefficient (r) = 0.994, apparent K_D = 0.26 nM, and receptor density (B_{max}) = 680 fmol/mg membrane protein for the specific binding of (³H)DHA to rat lung membranes.

$$k_1 = \frac{k_{obs} - k_{-1}}{L}$$

The second equation [2] could be used since no more than 10% of the initial free ligand was bound at steady state.

The dissociation rate constant (k_{-1}) was estimated by incubating radioligand and lung membranes to a steady state and then adding more than a 1,000-fold excess of unlabeled (−)-propranolol. Thus, as each molecule of [³H]DHA dissociates from its receptor, the probability of unlabeled (−)-propanolol reassociating in its place is more than 1,000 times that before the addition of unlabeled (−)-propranolol. Receptor bound [³H]DHA was assayed at various time

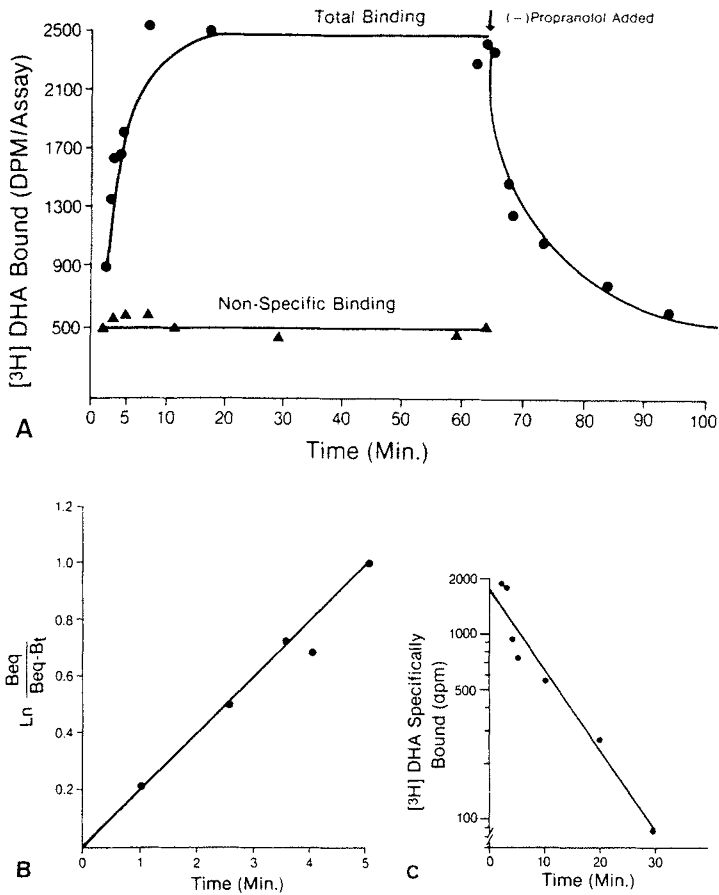

Fig. 3A. Rate of association and dissociation of [^{3}H]DHA binding to rat lung membranes as a function of time. Total binding was determined in the absence of (–)-propranolol, nonspecific binding was measured in the presence of 1 μM (–)-propranolol. In a parallel study, (–)-propranolol was added to the incubation mixture of tissue and radioligand after 70 min of incubation and total bindng and dissociation of [^{3}H]DHA from the receptor was monitored for an additional 60 minutes. The concentration of [^{3}H]DHA was 0.27 nM and the indubation mixture contained the equivalent of 0.05 mg/ml lung tissue. The curve shown is representative of three trials under similar conditions. **B** The rat of association of specific [^{3}H]DHA binding to rat lung membranes. The $L_n \frac{B_{eq}}{B_{eq} - B_t}$ was plotted vs. time [2]. B_{eq} is specific [^{3}H]DHA binding at equilibrium and B_t is specific binding of [^{3}H]DHA at time, t. **C** Rate of dissociation of specific [^{3}H]DHA binding to rat lung membranes. Data of **A** were plotted semilogarithmically vs. time. $t_{1/2}$ = 7 min.

intervals thereafter (Fig. 3A). At 25°C, the dissociation appeared to be a first-order process represented by a straight line with a slope, $k_{-1} = 0.098\ min^{-1}$ (Fig. 3C). Substituting in the equation

$$k_1 = \frac{k_{obs} - k_{-1}}{L} \text{ where } k_{obs} = 0.19\ min^{-1},\ k_{-1} = 0.098\ min^{-1}$$

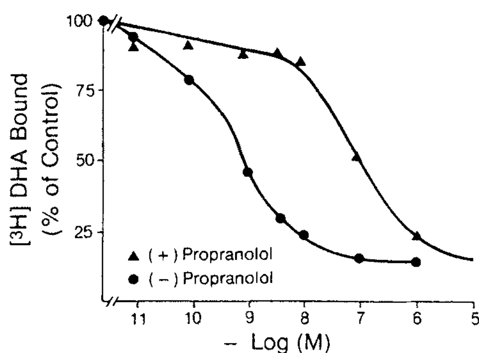

Fig. 4. Inhibition of specific [^{3}H]DHA binding by the stereoisomers of propranolol. [^{3}H]DHA concentration was 0.45 nM; protein concentration was 0.05 mg/ml. The curves show for each isomer the mean of four trials performed in duplicate. The SEM of each point was less than ± 5%.

and L = 0.27 nM; then $k_1 = 0.3407\ \text{min}^{-1}\ \text{mM}^{-1}$.

$$K_D = \frac{k_{-1}}{k_1} = \frac{0.098\ \text{min}^{-1}}{0.3407\ \text{min}^{-1}\ \text{nM}^{-1}} = 0.27\ \text{nM},$$

which is in close agreement with the value for K_D obtained from the previous saturation study.

Stereoselectivity of Beta-Adrenergic Receptor in Lung Tissue

The pattern of (−)- and (+)-propranolol inhibition of specific [^{3}H]DHA binding to rat lung tissue was used to determine whether the adrenergic receptor in this tissue was stereospecific. Figure 4 shows an inhibition experiment in which the concentration of (−)- and (+)-propranolol inhibiting specific binding of [^{3}H]DHA by 50%, (the IC_{50}) was estimated. By interpolation from the curves shown in Figure 4, the concentration of (−)- and (+)-propranolol inhibiting the specific binding of [^{3}H]DHA was 7.9×10^{-10} M and 1×10^{-7} M respectively. The corresponding K_I values for (−)- and (+)-propranolol using the Cheng and Prussof (5) equation:

$$K_i = \frac{IC_{50}}{1 + \frac{(^3H)\ DHA}{K_D}}$$

are 0.2 nM and 36.63 nM respectively. These results strongly support the stereoselectivity of the beta-adrenergic receptor in this tissue.

Inhibition of [3H]DHA Binding by Bronchodilator Drugs

Lung membranes prepared as described previously were incubated with [^{3}H]DHA at 0.4 to 0.5 nM concentrations with and without 1 μM (−)-proprano-

Table 2. Inhibition of [^{3}H]DHA binding to rat lung tissue by bronchodilator drugs

Compound	IC_{50} ± SE (M) × 10^{-7}	K_i ± SE (M) × 10^{-7}
Terbutaline	33.1 ± 9.34	13.2 ± 3.7
Albuterol	14.9 ± 1.70	5.9 ± 0.68
Procaterol	1.62 ± 0.28	0.65 ± 0.11
Isoproterenol	1.36 ± 0.53	0.54 ± 0.21
Zinterol	0.51 ± 0.08	0.20 ± 0.03

The IC_{50} for each drug was determined as described in the text and Figure 5. Values given are the means ± SE of four assays with each done in duplicate. The K_i was calculated from the IC_{50} value using the Cheng and Prusoff equation [5]:

$$K_i = \frac{IC_{50}}{1 + \frac{(^3H)\ DHA}{K_D}}$$

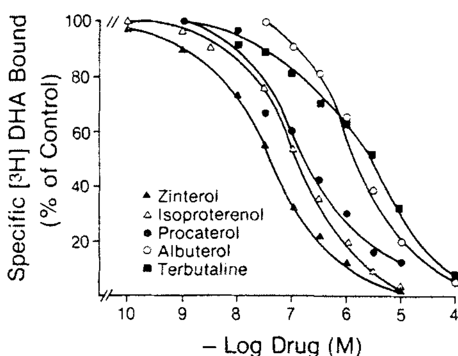

Fig. 5. Inhibition of [^{3}H]DHA binding to rat lung membranes by bronchodilator compounds. Curves represent one of four or more trials with each bronchodilator compound. Mean values for the IC_{50} ± SEM and the corresponding values for the K_i ± SEM for each compound studied are listed in Table 2.

lol to obtained control (maximum) specific [^{3}H]DHA binding. Separately, 10^{-10} to 10^{-4} M concentrations of the broncholdilator drugs shown in Table 2 were added to the incubation mixture containing radioligand. Tissue was then added and the reaction mixture incubated for 60 min at 25°C. Specific [^{3}H]DHA binding at each drug concentration for 1 of 4 or more trials with each bronchodilator compound is shown in Figure 5. IC_{50} values were interpolated from the curve for each assay for each of the 5 compounds studied.

Stimulation of Adenyl Cyclase Activity by (−)-Isoproterenol

The cAMP formed by rat lung tissue in 10 min at 37°C without agonist (the basal activity) was referred to the amount of cAMP formed by the lung tissue in the presence of 1 μM isoproterenol. The mean value of the agonist stimulated adenyl cyclase (5 trials) was 1.77 ± 0.06 times that of the basal (no agonist) adenyl cyclase activity (Table 3).

Table 3. Rat lung adenylate cyclase activity without and with (−)-isoproterenol

Trial[a]	Basal[b] activity	(−)Isoproterenol-[c] stimulated activity	Stimulation[d] due to (−)-Isoproterenol (%)
	(pmol/min/mg protein)		
1	45.3	89.1	96
2	51.3	92.9	81
3	85.6	146.0	71
4	59.0	109.1	85
5	68.6	107.7	57

[a] All trials in duplicate.
[b] No agonist present.
[c] 1 μM (−)-isoproterenol present during incubation for 10 min at 37°C.
[d] Percent mean stimulation ± SEM = 77.4 ± 6.4.

Discussion

Rugg et al. [16] prepared membranes from rabbit and rat lung with the aim of studying the proportions of $beta_1$ and $beta_2$ adrenoceptor binding sites which coexist within lung tissue of the 2 species.

We measured (^{3}H)DHA binding to membranes prepared from rat lung, heart, and skeletal muscle with the hope of developing an in vitro assay system to screen new compounds for potential bronchodilator activity.

Radioligand binding to membranes prepared from these tissues showed the number of receptor sites (B_{max}) in lung membranes to be approximately 7 and 8 times greater than in membranes prepared from rat heart and skeletal muscle, respectively (Table 1). The substantially greater binding of (^{3}H)DHA shown by lung membranes over membranes from heart and skeletal muscle confers greater sensitivity and accuracy in measuring the effects of inhibition of (^{3}H)DHA binding by the bronchodilator agonists shown in Table 2.

The rat lung membrane preparation also satisfied the criteria expected for adrenoceptor binding. Saturability and high affinity were demonstrated (Fig. 2A, B). The kinetic data for association and reversibility of (^{3}H)DHA binding extend the earlier work by Rugg et al. [16] and serve to characterize the adrenoceptor properties of rat lung membrane in greater detail (Fig. 3). (−)-propranolol was 180 times more effective in displacing specifically bound (^{3}H)DHA than (+)-propranolol indicating stereospecificity (Fig. 4).

The competition for binding of (^{3}H)DHA by the agonists studied is related to their intrinsic activity for beta-adrenergic receptors and helps to explain their pharmacological activity in the lung. For example, isoproterenol, a relatively nonselective bronchodilator, is a potent inhibitor of [^{3}H]DHA binding to the rat lung membrane preparation ($K_i = 0.54 \times 10^{-7}$ M). Procaterol and zinterol were examined for potency as inhibitors of (^{3}H)DHA binding to the rat lung membrane preparation since these compounds were shown to have a high affinity for $beta_2$ adrenoceptors [7, 13]. Our data for the inhibition of the specific binding of (^{3}H)DHA by procaterol ($K_i = 0.65 \times 10^{-7}$ M) are comparable with those

of Hazeki and Ui [7] who used rat lung membranes to correlate inhibition of (^{3}H)DHA binding by a number of agonists with effects upon adenylate cyclase activity. In addition, our value for the K_i of zinterol obtained from its capacity to inhibit the specific binding of (^{3}H)DHA ($K_i = 0.2 \times 10^{-7}$ M) is in good arrangement with that of Minneman et al. [13] obtained from the specific inhibition of binding of (^{125}I)-iodohydroxypindolol, ($K_i = 0.4 \times 10^{-7}$ M).

In our rat lung membrane preparation the order of potency of bronchodilator compounds studied was: zinterol > isoproterenol ≧ procaterol > albuterol ≧ terbutaline (Table 2). Comparative preliminary results using our rat skeletal muscle membranes to determine IC_{50} for displacement of specifically bound (^{3}H)DHA by isoproterenol, procaterol and zinterol were 7.5×10^{-7} M, 17.8×10^{-7} M, and $\geqq 20.0 \times 10^{-7}$ respectively (Fleisher JH, Pinnas JL: unpublished observations). The order of potency of these agonist compounds in skeletal muscle membranes was isoproterenol > procaterol ≧ zinterol, indicating that isoproterenol had greater affinity for muscle membranes than the latter 2 drugs. Minnemann et al. [14] have reported that $beta_2$ adrenoceptors are present in skeletal muscle. Inasmuch as muscle tremor in humans induced by sympathomimetic drugs appears to be mediated by beta adrenoceptors [9, 19], displacement of (^{3}H)DHA binding in skeletal muscle membranes may help to evaluate bronchodilator agonists for their potential tremorgenic effects.

Although comparison between radioligand binding studies and those on isolated tissues was not part of thc study, it is relevant that the displacement of (^{3}H)DHA in rat lung membranes by albuterol and terbutaline (Table 2) agrees with the observations of O'Donnell [15] that these compounds showed a greater potency in vitro on isolated guinea pig trachea than on isolated atria, a property which is associated with the $beta_2$ selectivity of these drugs.

Lung $beta_2$-adrenergic receptors appear to mediate bronchial relaxation through the production of cyclic AMP [10]. The finding that the adenylate cyclase activity of our preparation substantially increased when incubated with (−)-isoproterenol (Table 3) indicates the biochemical functionality of the rat lung preparation.

Our observations suggest that displacement of specific (^{3}H)DHA binding from rat lung and skeletal muscle membranes may provide useful data in screening new compounds for selective bronchodilator activity.

Acknowledgment. The authors thank Dr. HI Yamamura of the Department of Pharmacology, Arizona Health Sciences Center, for his helpful discussions and criticism of the manuscript; and Dr. G. Meinke of the Departments of Internal Medicine and Microbiology, Arizona Health Scineces Center, for assistance with adenyl cyclase assay.

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Accepted for publication: February 4, 1985