Solubilization and Characterization of the β-Adrenergic Receptor Binding Sites of Frog Erythrocytes

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Specific β-adrenergic receptors present in membrane preparations of frog erythrocytes were identified by binding of (-)-[3H]dihydroalprenolol, a potent competitive β-adrenergic antagonist. The (-)-[3H]dihydroalprenolol binding sites could be solubilized by treatment of a purified erythrocyte membrane fraction with the plant glycoside digitonin but not by treatment with a wide variety of other detergents. The binding sites appeared to be soluble by several independent experimental criteria including (a) failure to sediment at 105,000 x g for 2 hours; (b) passage through 0.22 μm Millipore filters; (c) chromatography on Sepharose 6B gels; and (d) electron microscopy. The soluble receptor sites retained all of the essential characteristics of the membrane-bound sites, namely rapid and reversible binding of β-adrenergic agonists and antagonists; strict stereospecificity toward both β-adrenergic agonists and antagonists; appropriate structure-activity relationships; saturability of the sites at low concentrations of ligand; no affinity for α-adrenergic drugs, nonphysiologically active catechol compounds, and catecholamine metabolites. Based on gel chromatography in the presence of detergent, the molecular weight of the soluble receptor is estimated to be no greater than 130,000 to 150,000.

Equilibrium binding studies indicated a Kd for the soluble receptor of 2 nM. Hill coefficients (nH) of 0.77 and curved Scatchard plots suggested the presence of negatively cooperative interactions among the solubilized receptors in agreement with previous findings with the membrane-bound sites. Kinetic studies indicated an association rate constant k1 = 3.8 x 10^6 M⁻¹ min⁻¹ and a reverse rate constant k₂ = 2.3 x 10⁻³ min⁻¹ at 4°C. The kinetically derived Kd (k₂/k₁) of 0.6 nM is in reasonable agreement with that determined by equilibrium studies.

The soluble receptors were labile at temperature > 4°C but could be stabilized with high concentrations of EDTA. Guanidine hydrochloride and urea produced concentration-dependent losses of binding activity which were partially reversible upon dialysis. Trypsin and phospholipase A both degraded the soluble receptors but a variety of other proteases and phospholipases as well as DNase and RNase were without effect. Experiments with group-specific reagents indicated that free lysine, tryptophan, serine, and sulfhydryl groups may be important for receptor binding. These studies suggest that the receptor is probably a protein which requires lipids for functional integrity. Data obtained with the solubilized binding sites are consistent with the contention that these sites represent the physiologically relevant β-adrenergic receptors which have been extracted from the membranes with full retention of their properties.

β-Adrenergic receptors are those components of certain catecholamine-sensitive cells which recognize and bind these drugs. Drug binding leads to generation of a signal which activates a biological process (1, 2). In many tissues the β-adrenergic receptors are linked to the enzyme adenylate cyclase (3). This is the case in avian (4-7) and amphibian erythrocytes (8-11). Until recently, the β-adrenergic receptors had not been directly identified. In the last year, however, several groups have demonstrated the feasibility of studying directly the β-adrenergic receptors with radioactively labeled β-adrenergic antagonists (12-17). We have used (-)-[3H]dihydroalprenolol, a potent competitive β-adrenergic antagonist, labeled to a specific activity of 17 to 33 Ci/mmol to identify binding sites in frog erythrocyte membranes which possess all of the essential characteristics to be expected of the physiologically β-adrenergic receptors. The membrane-bound receptor sites bind β-adrenergic agonists and antagonists rapidly and reversibly with affinity constants which are directly parallel to the known β-adrenergic potency of these agents (13, 16, 18). The binding of β-adrenergic agents moreover displays marked stereospecificity. There are approximately 1500 receptor sites per frog erythrocyte (16). The structure-activity relationships...
which determine the binding affinity of β-adrenergic agents for these sites have been extensively examined (18).

As with other adenylyl cyclase-coupled receptors, the β-adrenergic receptors are membrane-bound. Thus, in order to further characterize the nature of these receptors it was necessary to develop procedures for their solubilization from membranes. In this paper, we report (a) the successful solubilization of the β-receptors ((-)-[3H]dihydroalprenolol binding sites) from a purified membrane fraction derived from frog erythrocytes by treatment with digitonin; (b) a convenient chromatographic assay method which permits study of these sites in the soluble state; (c) data which suggest the identity of the soluble sites with the membrane-bound β-adrenergic receptors; and (d) kinetic, specificity, and other properties of the β-adrenergic binding sites in the solubilized state.

EXPERIMENTAL PROCEDURE

Materials

(-)- and (+)-Alprenolol hydrochloride were from Hassle. (-)- and (+)-Propranolol hydrochloride were from Ayerst. The (-)-isomers of isopropenol hydrochloride, epinephrine bitartrate, and norepinephrine bitartrate were from Winthrop. The (+)-isomers of isopropenol hydrochloride, epinephrine bitartrate, norepinephrine bitartrate, as well as dihydroxymercadic acid, (-)-dihydroxyphenylalanine, (+)-propranolol, cyclic AMP*, ATP, phosphoglycerate mutase, myokinase, and digitonin (lots D43B-335, D61B-069, and 87B-3229) were from Sigma. Digitonin (lot 745499) was also obtained from Fisher; lycoscinth is from General Biochemicals. Pervane kinase was from Calbiochem. [3H]Cyclic AMP (1 to 5 Ci/mmol) and [3H]ATP (10 to 20 Ci/mmol) were from New England Nuclear. Alumina, neutral grade was from ICN and Dowex 50W-X6 was from Bio-Rad. Sephadex G-50 were from Pharmacia. Sources and specific activities of enzymes used were as follows: phosphatase A (be venom, 1180 units/mg); phosphatase D (cabbage, 22 units/mg); DNase (beef pancreas, 2100 kilounits/mg); RNase A (bovine pancreas, 100 kilounits/mg); trypsin (type II, 2000 units/mg), and papain (hog stomach, 4500 units/mg) were from Sigma. Theolysin (6500 picounits/mg) was obtained from Nasco-Steinhilber.

(-)-Alprenolol was solubilized at New England Nuclear by catalytic reduction with tritium gas using palladium as the catalyst, to a specific activity of 17 to 33 Ci/mmol. (-)-Alprenolol was also reduced at New England Nuclear with hydrogen gas using identical procedures. Both materials were purified so that they were homogeneous in six different thin layer chromatography systems (18). As described elsewhere, in each of these systems the tritiated, hydrogenated, and native alprenolol had identical Rf values (18).

When chromatography is performed on silica gel plates impregnated with 5% silver nitrate ("argentation" chromatography), compounds differing by only a single unsubstated bond may be separated (50). When labeled material was chromatographed on such plates and compared with native (-)-alprenolol the following results were obtained: (a) acetone/benzene/acetic acid, 79/2/5; (b) alprenolol Rf, 0.65; tritiated alprenolol and hydrogenated alprenolol Rf, 0.58; (b) methanol/benzene/water, 15/25/8; (c) alprenolol Rf, 0.4; tritiated alprenolol and hydrogenated alprenolol Rf, 0.83.

The abbreviations used are: cyclic AMP, adenosine 3'-5'-monophosphate; GuHCl, guanidine hydrochloride.

The fact that the specific activity of the labeled material is less than "theoretical" (i.e., ~6 Ci/mmol) is presumably due to tritium exchange with solvent during the labeling procedure which leads to reduction of some double bonds with hydrogen rather than tritium atoms. It is not due to the presence of unreduced double bonds in the labeled material.

We have tested the biological activity of (1)-dihydroalprenolol, (-)-alprenol, and several lots of the radiolabeled material with specific radioactivities ranging from 10 to 33 Ci/mmol. When these materials were tested as antagonists of isoproterenol-stimulated adenylate cyclase by previously described procedures (16, 18) the Rf of all of these materials was identical, ~0.5. Since the tritiated material contains no unreacted native (-)-alprenol, these assays represent valid potency estimates of the tritiated compound. The results also indicate the biological equivalence of (1)-alprenol and (1)-dihydroalprenol.

This equivalence was further tested by comparing the ability of (1)-dihydroalprenolol and (-)-alprenol to compete with "(-)-[3H]dihydroalprenolol" for binding sites in frog erythrocytes. The two materials gave identical displacement curves, further indicating their identical biological activity.

Methods

Membrane Preparations—Heparinized blood from grass frogs maintained at room temperature was collected by cardiac puncture and erythrocytes were washed three times with a solution of 110 mm NaCl, 5 mm KCl, 2 mm CaCl2, 1 mm MgCl2, which had been repeatedly washed with a buffer: 25 mM Tris-HCl, pH 7.4, 130 mm NaCl, and centrifuged at 30,000 x g for 30 min. A crude erythrocyte membrane preparation was obtained by washing this pellet twice and resuspending the material in 75 mm Tris-HCl, pH 8.1/25 mm MgCl2. To obtain the purified membrane preparation, the pellet of the first centrifugation was resuspended in 10 mm Tris-HCl, pH 8.1/10 mm MgCl2 by homogenization and centrifuged at 2,000 x g for 10 min over a cushion of the same buffer containing 50% sucrose. The material which sedimented through sucrose was discarded and the supernatant was centrifuged at 30,000 x g for 15 min. The pellet was washed once with 5 mm Tris-HCl, pH 8.1/25 mm MgCl2 for adenylate cyclase and (-)-[3H]dihydroalprenolol binding assays. For solubilization experiments the final pellet was resuspended in 50 mm Tris-HCl, pH 7.4, containing digitonin or other detergents. Membranes were usually prepared fresh prior to each experiment. All procedures were performed at 0-4°C.

Subsolubilization Procedures—Purified erythrocyte membranes (preparations described above) were suspended in 50 mM Tris-HCl, pH 7.4, generally containing 1% digitonin (4.5 to 5.5 mg of membrane protein/3 ml of 1% digitonin buffer). The suspension was allowed to stand in ice for 30 to 40 min with periodic agitation and was then centrifuged in a Sorval RC2-B centrifuge at 30,000 x g for 30 min. The clear yellowish supernatant constituted the solubilized preparations which have been used throughout the studies reported here. This centrifugation procedure was routinely used since more prolonged centrifugation at higher g forces did not sediment additional material (see "Results").

Adenylate Cyclase Assays—Adenylate cyclase assays were performed as described previously (11) and cyclic [3H]AMP was isolated according to the method of Salton et al. (19).

Binding Assays—(-)-[3H]Dihydroalprenolol binding in membrane fractions was assayed by a centrifugal assay as described previously (16, 18). (-)-[3H]Dihydroalprenolol, ~25 nm, was incubated with 0.3 to 0.4 mg of membrane protein for 10 min at 37° in a medium containing 50 mm Tris-HCl, pH 7.4, and 15 mm MgCl2 in a volume of 150 μl. At the completion of the incubations, duplicate 10-μl aliquots were placed over 300 μl of the incubation buffer in small polyethylene centrifuge tubes and centrifuged for 1 min in a Beckman Microfuge 152. The membranes and bound (-)-[3H]dihydroalprenolol were pelleted almost immediately. The surface of the pellet was washed once with the same buffer, and the pellet was solubilized overnight by shaking with 0.5 ml of 10% sodium dodecyl sulfate and 300 mM EDTA and then counted in a liquid scintillation spectrometer after addition of a Triton X-100 toluene-based fluor. In all experiments the amount of (-)-[3H]dihydroalprenolol "nonspecifically" bound and/or trapped in the membrane pellets was determined by incubating membranes and (-)-[3H]dihydroalprenolol in the presence of 10 μM (+)-alprenol or...
propranolol which blocked all β-adrenergic receptor binding sites (16, 18). Although the choice of concentration of unlabeled ligand used to determine nonspecific binding is always somewhat arbitrary, we selected 10 μM propranolol for several reasons. Higher concentrations of ligand do not displace additional radioactivity. Using this concentration of ligand, calculated Kᵦ values from binding experiments for numerous ligands are in excellent agreement with Kᵦ values determined from adenylate cyclase experiments (18). "Nonspecific" binding was generally 10 to 15% of total binding and was subtracted from all experimental values. "Specific binding" in purified membrane preparations ranged from 2 to 2.5 pmol of (±)[3H]dihydroalprenolol bound/mg of protein, at saturating concentrations of radioligand.

This binding method has been validated by equilibrium dialysis. In three experiments, with (±)[3H]dihydroalprenolol present at 1 x 10⁻⁸, specific binding as assessed by the centrifugation method was 1.30 ± 0.2 pmol/mg of protein, and by equilibrium dialysis (18 hours, 4°C) 1.37 ± 0.14 pmol/mg of protein (not statistically significant). Thus, binding as assessed by this simple centrifugal assay is a true reflection of equilibrium binding. It should be noted that the half-time for dissociation of (±)[3H]dihydroalprenolol from the receptors (<2 min at 37°C; -5 min at room temperature at which centrifugation is performed) is sufficiently slow relative to the time needed to pellet the membranes (a few seconds) so that insignificant dissociation occurs during the procedure (16, 51).

Equilibrium Dialysis—Membrane preparations solubilized with digitonin (500 to 700 μg of protein) were dialyzed in the presence or absence of various drugs and hormones against digitonin solutions containing 50 mM Tris-HCl, pH 7.4, at 4°C, and (±)[3H]dihydroalprenolol at a concentration of 10 nM in a total volume of 2 ml. The dialysis cells were rotated for 16 to 20 hours at 4°C. Quadruplicate 200-μl samples were removed from both sides of the dialysis cells and counted in toluene-Triton X-100 scintillation fluid. The difference in radioactivity between the sample side and the medium side of cells was taken as "total" binding. "Specific" binding was determined in each experiment as the difference between total binding and the binding which occurred in the presence of 10 μM (+)-propranolol or unlabeled (±)-alprenolol ("nonspecific" binding). Nonspecific binding in the preparations obtained with 1% digitonin varied from 20 to 45% of the total binding.

Sephadex G-50 Chromatographic Procedure—Aliquots (200 μl) of solubilized preparations (50 mM Tris-HCl, pH 7.4, at 4°C, 1% digitonin) were incubated at 4°C for 90 min with 30 to 40 mM (±)[3H]dihydroalprenolol in a total volume of 250 μl. At the end of the incubation, samples were diluted to 500 μl with 50 mM Tris-HCl, pH 7.4, and chromatographed at once on Sephadex G-50 columns (0.6 x 12 cm) at 4°C. Samples were loaded on top of the pre-equilibrated columns which were eluted with 50 mM Tris-HCl, pH 7.4, by gravity. Buffer, 0.5 ml, was applied to the column and the eluate was discarded. An additional 1.2 ml fraction contained the "void volume" of the column as determined with blue dextran 2000 as a marker and contained the "bound" (±)[3H]dihydroalprenolol. At the completion of the chromatography the columns were washed with 20 ml of the same buffer and stored in buffer containing sodium azide (0.05%). The same columns can be reused for 10 to 15 mins. (±)[3H]dihydroalprenolol specific binding was determined from the difference between total binding and the binding obtained in the presence of 10 μM (+)-propranolol or alprenolol (nonspecific binding). Specific binding in solubilized preparations as assessed by chromatography was greater than 95% of the total binding. As shown under "Results" (Fig. 4), dissociation of bound (±)[3H]dihydroalprenolol from the sites was negligible during the 15 to 20 min required to complete the chromatography.

Both methods of assessing binding of (±)[3H]dihydroalprenolol gave very comparable results. Table I shows a comparison of the two methods. (±)[3H]dihydroalprenolol binding assessed in solubilized preparations by equilibrium dialysis was slightly lower than that measured by the chromatographic procedure. This difference may be attributable to the relative instability of the solubilized binding sites during the prolonged incubations necessary for the equilibrium dialysis procedure. In addition to being a much more rapid method, the chromatography on Sephadex G-50 gave a higher percentage of "specific binding" (95 to 100%).

Protein—Protein determinations were carried out by the method of Lowry et al. (20) using bovine serum albumin as the standard. Estimations of samples containing digitonin were corrected for the presence of digitonin.

**RESULTS**

**Preparation of Purified Erythrocyte Membranes—**Lysates prepared from amphibian erythrocytes contain large amounts of nuclear material as well as other elements. In order to facilitate solubilization of the (±)[3H]dihydroalprenolol binding sites, a purified membrane fraction was prepared. As shown in Table II, centrifugation of a crude erythrocyte preparation over a 50% sucrose cushion removed a large portion of the protein while affording a considerable increase in the specific activity of the membrane-bound adenylate cyclase and the (±)[3H]dihydroalprenolol binding activity. Basal adenylate cyclase activity showed a 4.3-fold increase in specific activity.

**TABLE I**

Comparison of equilibrium dialysis with chromatography on Sephadex G-50 for assessment of (±)[3H]dihydroalprenolol binding to solubilized preparations

<table>
<thead>
<tr>
<th>Method</th>
<th>Specific binding</th>
<th>nonspecific binding</th>
<th>% total binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium dialysis</td>
<td>0.210 ± 0.045</td>
<td>44 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-50 chromatography</td>
<td>0.233 ± 0.005</td>
<td>5.5 ± 0</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

Purification of a membrane fraction from frog erythrocytes

The various fractions were prepared as described under "Methods." (±)[3H]dihydroalprenolol binding was assayed by a centrifugation method. n = the number of preparations tested. All results were determined in duplicate. Values shown are mean ± S.E.M. Numbers in parentheses indicate the percentage recovery of the adenylate cyclase and (±)[3H]dihydroalprenolol binding activities.

<table>
<thead>
<tr>
<th>Fractious</th>
<th>Protein</th>
<th>Adenylate cyclase (±)[3H]dihydroalprenol bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>crude erythrocyte</td>
<td>11.9 ± 0.2</td>
<td>7.09 ± 240 (100)</td>
</tr>
<tr>
<td>50% sucrose pellet</td>
<td>11.6 ± 1.3</td>
<td>37.5 ± 11.2 (59)</td>
</tr>
<tr>
<td>purified membrane</td>
<td>1.9 ± 0.4</td>
<td>3070 ± 600 (69)</td>
</tr>
</tbody>
</table>
and retained catecholamine responsiveness. (−)-[^3H]Dihydroalprenolol binding activity in the final membrane fraction was purified 9.5-fold. (−)-[^3H]Dihydroalprenolol binding sites in this purified fraction possessed the same characteristics (18, 21, 22) as the sites studied in unfractionated preparations (13, 16). The fact that recovery of binding activity is greater than 100% in the purified membrane fractions may be due to loss of some inhibitory factor during the preparation.

Solubilization of (−)-[^3H]Dihydroalprenolol Binding Sites—Treatment of the purified erythrocyte membrane fractions with the plant glycoside digitonin released the membrane-bound (−)-[^3H]dihydroalprenolol binding sites in a solubilized form to a degree dependent on the concentration of digitonin (Table III). Solubilization of erythrocyte membranes with 1% digitonin was used routinely throughout the studies reported here. The solubilization of the (−)-[^3H]dihydroalprenolol binding sites by digitonin appeared to be quite specific for this detergent since a number of other detergents were ineffective in solubilizing the binding sites in an active form. The detergents tested were Lubrol PX, 0.2% 1%; Lubrol WX, 1%; Triton X-100, X-305, N-101, CF-54 at 1%; sodium deoxycholate and octyl sodium sulfate at 0.1, 0.25, 0.5, and 1%. Lithium diiodosalicylate, lysolecithin, phospholipase A, and EDTA were also tested and found to be ineffective in releasing the binding sites in an active form. These data do not rule out the possibility that certain detergents did solubilize the sites but also interfered with the binding assay.

To verify the soluble state of the (−)-[^3H]dihydroalprenolol binding sites released by treatment with digitonin, we subjected solubilized preparations to a number of experimental tests, namely, ultracentrifugation, Millipore filtration, gel filtration, and electron microscopy. When 1% digitonin-solubilized preparations obtained by centrifugation at 30,000 × g for 30 min were subjected to further ultracentrifugation at 105,000 × g for 2 hours, no sedimented material was observable and (−)-[^3H]dihydroalprenolol binding remained unchanged. In addition, after passage of the preparations through 0.45-μm and 0.22-μm filters, no decrease in binding was observed.

When solubilized preparations were fractionated by molecular sieving on Sepharose 6B, the (−)-[^3H]dihydroalprenolol binding activity was well included in the gel with a K, of about 0.6 (Fig. 5). In addition, electron microscopy indicated the total absence of any recognizable membrane structure in the solubilized preparations.

After treatment of membranes with digitonin, adenylate cyclase activity is present in the solubilized preparations at a specific activity comparable to that observed in the membrane fraction (membrane: 280 pmol/min/mg; soluble: 245 pmol/min/mg, comparison of four experiments). The solubilized enzyme, however, no longer responds to catecholamine stimulation (data not shown).

The (−)-[^3H]dihydroalprenolol binding activity present in solubilized preparations was stable to storage at −38° for at least 1 month. Binding activity assessed either by equilibrium dialysis or chromatographic techniques was linearly related to the amount of protein over the range tested (0.05 to 1 mg/ml).

Stereospecific Binding Interaction of Adrenergic Agents and Other Agents with the Solubilized Sites—Fig. 1 demonstrates the ability of several adrenergic antagonists to compete with (−)-[^3H]dihydroalprenolol for binding to solubilized sites. As in the intact membrane (13, 16), the unlabeled β-adrenergic antagonists (−)-alprenolol and (−)-propranolol appear to be equipotent in inhibiting the binding of (−)-[^3H]dihydroalprenolol. The data also indicate that the solubilized sites retain their stereospecificity in binding β-adrenergic antagonists. The (−)-isomers of alprenolol and propranolol are about 2 orders of magnitude more potent than the respective (+)-isomers. The racemic compound (+)-dichlorisoproterenol, as expected, competed for the binding sites with a lesser potency than either (−)-alprenolol or (−)-propranolol. The solubilized binding sites show no affinity for the α-adrenergic antagonist phenolamine. The dissociation constants (K,) calculated from competition experiments (Fig. 1) (23) correlate very well with those previously delineated for the binding sites in intact frog erythrocyte membranes (18) (Table IV) even though the two sets of experiments were performed at quite different temperatures (4° soluble, 37° particulate). Table IV lists the calculated dissociation constants (K,) of several antagonists for the solubilized receptor sites in comparison to those obtained for the membrane-bound binding sites.

Fig. 2 demonstrates the ability of various adrenergic agonists to compete with (−)-[^3H]dihydroalprenolol for binding to the solubilized sites. These data indicate that strict stereospecificity is also apparent in the binding interaction of β-adrenergic agonists with these sites. The (−)-isomers of isoproterenol, epinephrine and norepinephrine are considerably more potent than the (+)-isomers of these compounds. Table V summarizes the stereoisomeric potency ratios for several agonists and antagonists obtained in the solubilized preparations and compares them with those obtained with the membrane-bound

### Table III

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein Yield</th>
<th>(−)-[^3H]Dihydroalprenolol bound</th>
<th>Yield % binding sites solubilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified membrane fraction</td>
<td>1700 ± 184</td>
<td>2.47 ± 0.37</td>
<td>1.45 ± 0.21</td>
</tr>
<tr>
<td>Solubilized preparations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% digitonin</td>
<td>105 ± 4.9</td>
<td>0.629 ± 0.028</td>
<td>2.76 ± 0.275</td>
</tr>
<tr>
<td>0.5% digitonin</td>
<td>325 ± 37.8</td>
<td>0.778 ± 0.120</td>
<td>2.39 ± 0.130</td>
</tr>
<tr>
<td>0.5% digitonin</td>
<td>561 ± 39.8</td>
<td>2.02 ± 0.20</td>
<td>3.60 ± 0.36</td>
</tr>
<tr>
<td>1.0% digitonin</td>
<td>846 ± 66.3</td>
<td>2.09 ± 0.20</td>
<td>2.47 ± 0.24</td>
</tr>
</tbody>
</table>

Solubilization of (−)-[^3H]Dihydroalprenolol binding sites from purified frog erythrocyte membranes

The purified membrane fraction was prepared as described under "Methods." Membrane protein, 4.5 to 5.5 mg, was suspended in 3 ml of 50 mM Tris-HCl buffer, pH 7.4, containing the indicated concentrations of digitonin. (−)-[^3H]Dihydroalprenolol binding in solubilized preparations was assessed by equilibrium dialysis. The results shown are the mean of quadruplicate determinations from three experiments ± S.E.
Soluble β-Adrenergic Receptors

Fig. 1. Competition of adrenergic antagonists for the binding of (-)-[3H]dihydroalprenolol to solubilized erythrocyte membrane preparations. (-)-[3H]Dihydroalprenolol (10 nM) binding was determined by equilibrium dialysis and gel chromatography in the presence and absence of the indicated concentrations of the various antagonists as described under "Methods." One hundred percent control binding was 2.46 ± 0.18 pmol/mg. The results shown are the mean of quadruplicate determinations from two experiments.

Fig. 2. Competition of β-adrenergic agonists and other agents for the binding of (-)-[3H]dihydroalprenolol to solubilized erythrocyte membrane preparations. (-)-[3H]Dihydroalprenolol binding was determined by equilibrium dialysis and gel chromatography in the presence and absence of various concentrations of these agents. (-)-[3H]Dihydroalprenolol was present in the assays at 10 nM. One hundred percent control binding was 1.42 ± 0.04 pmol/mg. Results shown are the mean of quadruplicate determinations from two experiments.

Table IV

Dissociation constants (Kd) of potent β-adrenergic antagonists for the solubilized (-)-[3H]dihydroalprenolol binding sites

Values for Kd were calculated according to Cheng and Prusoff (23) for competition experiments shown in Fig. 1. Data for the membrane-bound receptors were taken from Mukherjee et al. (18).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>(-)-[3H]Dihydroalprenolol Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Propranolol</td>
<td></td>
<td></td>
<td>0.0046 ± 0.0003</td>
</tr>
<tr>
<td>(+)-Propranolol</td>
<td></td>
<td></td>
<td>0.0037 ± 0.0005</td>
</tr>
<tr>
<td>(+)-Alprenolol</td>
<td></td>
<td></td>
<td>0.0034 ± 0.0002</td>
</tr>
<tr>
<td>(-)-Alprenolol</td>
<td></td>
<td></td>
<td>0.0041 ± 0.0002</td>
</tr>
<tr>
<td>(-)-Ioproprenalol</td>
<td></td>
<td></td>
<td>0.0015 ± 0.0005</td>
</tr>
<tr>
<td>(+)-Ioproprenalol</td>
<td></td>
<td></td>
<td>0.0021 ± 0.0002</td>
</tr>
<tr>
<td>MJ7963-1</td>
<td></td>
<td></td>
<td>0.0075 ± 0.0001</td>
</tr>
<tr>
<td>(-)-Dichlor-isoproterenol</td>
<td></td>
<td></td>
<td>0.0037 ± 0.0007</td>
</tr>
</tbody>
</table>

It is also apparent from the data shown that all of the structure-activity relations which characterize the membrane-bound β-adrenergic receptors have been preserved in the solubilized preparations. In addition to the (-)-stereoisomeric configuration of the β-carbon hydroxyl the size of the substituent on the amino nitrogen is an important determinant of affinity for the receptor. (+)-Cc-34, which has an aromatic substituent on the amino nitrogen, is several-fold more potent than isoproterenol in inhibiting (-)-[3H]dihydroalprenolol binding. The same observation can be made by comparing (+)-soterenol and MJ7963-1. (-)-Isoproterenol and (+)-dichlorisoproterenol had very comparable affinity. As noted elsewhere (18) the ring substituents are more important in determining the intrinsic activity of adrenergic agents for stimulation of adenylate cyclase than the affinity for the receptors. The solubilized (-)-[3H]dihydroalprenolol binding sites did not interact with the physiologically inert catechol compounds (±)-dihydroxyphenylalanine and dihydroxymandelic acid, even at concentrations as high as 100 µM.

Equilibrium Studies of (-)-[3H]Dihydroalprenolol Binding in Soluble Preparations—Binding of (-)-[3H]Dihydroalprenolol to the solubilized sites was a saturable process (Fig. 3). (Nonspecific binding was only 1 to 2% of the total binding and

receptor sites (16, 18) and those from intact tissues obtained from the literature (24). A reasonable correlation exists between the three sets of data.

The potency series for the various agonists in inhibiting binding of (-)-[3H]dihydroalprenolol was (-)-isoproterenol > (-)-epinephrine > (-)-norepinephrine, which is typical of the β-adrenergic receptor. It is also apparent from these data that the solubilized receptor sites retain the characteristics of a β2-adrenergic receptor (25). This is demonstrated by the fact that (-)-isoproterenol is 2 to 3 orders of magnitude more potent than (-)-norepinephrine in competing for the binding sites. In general, the affinities of agonists ((-)- and (+)-isomers) for the solubilized receptor sites correlate reasonably with the affinity of the binding sites for the same compounds in membrane preparations (Table VI).
TABLE V
Comparison of the stereoisomeric potency ratios of (-)- and (+)-\(\beta\)-adrenergic agonists and antagonists for \(\beta\)-adrenergic receptors
The values for the physiological responses in intact tissues are taken from the literature (24). Values for stereoisomeric potency ratios in frog erythrocyte membrane fractions are taken from our previous work (18). Values for the solubilized preparations were determined from the data of Figs. 1 and 2. Value for propranolol marked with an asterisk (*) refers to antagonism of isoproterenol-induced tachycardia in the cat, - indicates that (+)-isomer of norepinephrine is too weak to calculate a valid potency ratio.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Intact tissues: guinea pig atrium cell tracheal muscle (24)</th>
<th>Membrane fractions</th>
<th>Solubilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potency ratio = [((-)-isomer) / [(+)-isomer]:</td>
<td>Adenylate cyclase</td>
<td>(-)-[(\text{H})]dihydroalprenol binding</td>
</tr>
<tr>
<td>Alprenolol</td>
<td>30</td>
<td>44</td>
<td>30</td>
</tr>
<tr>
<td>Propranolol</td>
<td>45</td>
<td>60</td>
<td>146</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>700-2300</td>
<td>450</td>
<td>325</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>53</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>20</td>
<td>-</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

TABLE VI
Dissociation constants (\(K_D\)) of \(\beta\)-adrenergic agonists for binding to solubilized receptor sites
\(K_D\) values were calculated (23) from data shown in Fig. 2. Data for the membrane-bound receptors were taken from Mukherjee et al. (18).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Membrane Bound Receptors</th>
<th>Solubilized Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Norepinehrne</td>
<td>49 \pm 2</td>
<td>4114</td>
</tr>
<tr>
<td>(-)-Norepinehrne</td>
<td>200</td>
<td>152 \pm 26</td>
</tr>
<tr>
<td>(-)-Epinephrine</td>
<td>4610.2</td>
<td>28 \pm 14</td>
</tr>
<tr>
<td>(-)-Epinephrine</td>
<td>1374</td>
<td>-</td>
</tr>
<tr>
<td>(-)-Isoproterenol</td>
<td>0.40 \pm 0.005</td>
<td>0.21 \pm 0.005</td>
</tr>
<tr>
<td>(+)-Isoproterenol</td>
<td>1.83 \pm 0.05</td>
<td>6.15 \pm 0.005</td>
</tr>
<tr>
<td>(+)-Eutrenol</td>
<td>2.41 \pm 0.00</td>
<td>0.69 \pm 0.0001</td>
</tr>
<tr>
<td>(+)-Eutrenol</td>
<td>2.41 \pm 0.00</td>
<td>0.69 \pm 0.0001</td>
</tr>
</tbody>
</table>

approximated blank values.) In five experiments a saturation value of 2.0 \pm 0.3 pmol/mg was obtained. Half-maximal saturation, which provides an estimate of the equilibrium dissociation constant (\(K_D\)) of (-)-[\(\text{H}\)]dihydroalprenolol for the solubilized binding sites, was 2.2 \pm 0.2 nM (n = 5). Data obtained by equilibrium binding were analyzed graphically by Hill plots.

Kinetic Studies of (-)-[\(\text{H}\)]Dihydroalprenolol Binding in Soluble Preparations—Binding of (-)-[\(\text{H}\)]dihydroalprenolol to the solubilized sites was time-dependent. With (-)-[\(\text{H}\)]dihydroalprenolol present at 37.4 nM specific binding at 4\(^\circ\) reached equilibrium between 40 and 90 min (Fig. 4A). The calculated second order association rate constant determined from the time course of binding of (-)-[\(\text{H}\)]dihydroalprenolol was \(k_1 = 3.8 \times 10^6\) M\(^{-1}\) min\(^{-1}\) (27). Fig. 4B shows the time course of dissociation of (-)-[\(\text{H}\)]dihydroalprenolol from the solubilized sites at 4\(^\circ\). Dissociation of (-)-[\(\text{H}\)]dihydroalprenolol is relatively slow at 4\(^\circ\), \(t_{1/2}\) about 5 to 6 hours. As pointed out earlier, this permits the Sephadex G-50 chromatographic method of determining specific binding of (-)-[\(\text{H}\)]dihydroalprenolol under equilibrium conditions. From the slope of the line in Fig. 4B a dissociation rate constant (\(k_d\)) of 2.3 \times 10^{-5} \text{ min}^{-1} can be calculated. An estimate of the dissociation constant (\(K_D = k_d/K_D\)) of 0.6 nM can be obtained from the kinetic rate constants. This value is in reasonable agreement with the estimate of \(K_D\) obtained by other methods of analysis (see above).

It seemed of interest to compare the kinetic characteristics of the soluble and particulate receptors. Lability of the soluble receptors at 37\(^\circ\) precluded analysis at this temperature. When kinetic studies were performed at 4\(^\circ\) with the particulate receptors, these results were obtained: \(k_1 = 6.6 \times 10^6\) M\(^{-1}\) min\(^{-1}\), \(k_2 = 6.6 \times 10^{-3}\) min\(^{-1}\), \(K_D = k_d/h_0 = 1\) nM. Thus, at 4\(^\circ\) the kinetics of the soluble and particulate receptors are fairly comparable.

### Gel Filtration of Solubilized Receptors—Solubilized preparations containing 1% digitonin were chromatographed on a Sepharose 6B column which was equilibrated with 50 mM Tris-HCl, pH 7.4, at 4\(^\circ\). The elution profile of a typical run is shown in Fig. 5. The (-)-[\(\text{H}\)]dihydroalprenolol binding activity was eluted as a major peak with a \(K_D\) of 0.58 to 0.60. A small peak of binding activity also appeared to be present in the void volume. The elution pattern of the solubilized receptor activity was the same whether the preparations were chromatographed on columns equilibrated with a buffer containing 0.1%
Fig. 3. Binding of (-)-[3H]dihydroalprenolol to solubilized erythrocyte preparations as a function of increasing concentration of ligand. Solubilized preparations (515 to 705 pg/ml) were incubated at 4°C with various concentrations of (-)-[3H]dihydroalprenolol (53 Ci/mmol) (20 to 200 nM) for 180 min. Bound and free (-)-[3H]dihydroalprenolol were separated by Sephadex G-50 column chromatography as described under "Methods." Specific (-)-[3H]dihydroalprenolol binding was calculated from the difference between total binding and binding in the presence of 10 μM of (-) propranolol (nonspecific binding). Saturation binding was 2.0 ± 0.3 pmol/mg (n = 5). Results shown are mean of duplicate determinations. This experiment was replicated five times. 

Upper inset, Hill plot of (-)-[3H]dihydroalprenolol binding to solubilized receptor sites. B indicates the amount of specific (-)-[3H]dihydroalprenolol specifically bound to sites at saturation (Fig. 3). Data shown are means of duplicate determinations. The experiment shown is typical of five such experiments. Lower inset, Scatchard plot of (-)-[3H]dihydroalprenolol binding to solubilized erythrocyte membrane preparations. Data shown are means of duplicate determinations and this plot is typical of five such experiments.
Soluble β-Adrenergic Receptors

Solubilization of the β-adrenergic receptors ((−)-[3H]dihydroalprenol binding sites) from a purified membrane fraction of frog erythrocytes was achieved by treatment with digitonin. Although digitonin is not a widely used detergent for the solubilization of membrane proteins, it was used for this purpose long ago in studies with rhodopsin (44) and more recently with the muscarinic cholinergic receptor (45). The truly soluble state of the digitonin-extracted receptor preparations was defined by the usual experimental criteria of gel filtration, electron microscopy, ultrafiltration, and ultracentrifugation. A variety of other detergents were quite ineffective in solubilizing the receptors in an active form.

Of the two methods used to assay (−)-[3H]dihydroalprenolol binding to the soluble receptors, equilibrium dialysis and gel filtration, the filtration method proved more satisfactory for several reasons. Thus, equilibrium dialysis routinely gave nonspecific binding values which ranged from 20 to 45%. By comparison, nonspecific binding with the gel filtration method was 0 to 5% of total binding and approximated the blank. Several other advantages of the gel filtration assay are that it is rapid; it is highly reproducible and variability between duplicate determinations, and brackets represent the range. B, dissociation of (−)-[3H]dihydroalprenolol from solubilized preparations of frog erythrocyte membranes. A solubilized preparation was equilibrated at 4° with (−)-[3H]dihydroalprenolol (35 nM) for 90 to 120 min. At time zero, (±)-propranolol was added to the incubation mixture to a final concentration of 10 μM. At the indicated times, 250-μl aliquots were removed and chromatographed on Sephadex G-50 columns to determine bound (−)-[3H]dihydroalprenolol as described under “Methods.” Initial binding refers to the amount of (−)-[3H]dihydroalprenolol bound at equilibrium prior to the addition of (±)-propranolol. The slope of the line provides an estimate of the dissociation rate constant k₂, at 2°. Results shown are from duplicate determinations, and brackets represent the range.

DISCUSSION

Solubilization of the β-adrenergic receptors ((−)-[3H]dihydroalprenol binding sites) from a purified membrane fraction of frog erythrocytes was achieved by treatment with digitonin. Although digitonin is not a widely used detergent for the solubilization of membrane proteins, it was used for this purpose long ago in studies with rhodopsin (44) and more recently with the muscarinic cholinergic receptor (45). The truly soluble state of the digitonin-extracted receptor preparations was defined by incubation of buffer (50 mM Tris-HCl, pH 7.4, containing 1% digitonin) with (−)-[3H]dihydroalprenolol followed by gel chromatography. Data shown are means of duplicate determinations, and brackets represent the range. B, dissociation of (−)-[3H]dihydroalprenolol from solubilized preparations of frog erythrocyte membranes. A solubilized preparation was equilibrated at 4° with (−)-[3H]dihydroalprenolol (35 nM) for 90 to 120 min. At time zero, (±)-propranolol was added to the incubation mixture to a final concentration of 10 μM. At the indicated times, 250-μl aliquots were removed and chromatographed on Sephadex G-50 to determine bound (−)-[3H]dihydroalprenolol as described under “Methods.” Initial binding refers to the amount of (−)-[3H]dihydroalprenolol bound at equilibrium prior to the addition of (±)-propranolol. The slope of the line provides an estimate of the dissociation rate constant k₂, at 2°. Results shown are from duplicate determinations, and brackets represent the range.

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Fig. 5. Sepharose 6B chromatography of solubilized (–)-[3H]dihydroalprenolol binding protein. A solubilized preparation (2.0 ml) concentrated to 4.2 mg/ml by ultrafiltration (Amicon PM-10) was chromatographed on a Sepharose 6B column (1.5 x 62 cm) at a constant flow rate of 10 ml/hour. The column was equilibrated with 40 mM Tris-HCl, pH 7.4, at 4°. Fractions of 2 ml were collected. Specific (–)-[3H]dihydroalprenolol binding (O––O) was assayed (35 nM (–)-[3H]dihydroalprenolol) on the various fractions as described under “Methods” for the Sephadex G-50 chromatographic assay. The protein elution profile (●–●–●) was determined spectrophotometrically at 280 nm. Arrows indicate the position of the peak protein elution when various marker proteins (5 to 15 mg/2 ml) were chromatographed on the same column under identical conditions. The results shown are representative of three experiments. Inset, estimation of the apparent molecular weight of solubilized (–)-[3H]dihydroalprenolol binding sites by gel filtration on Sepharose 6B (29). The curve was constructed by plotting the known molecular weights of the various marker proteins versus their relative elution volume (Kᵥ).
solubilized preparations was saturable. Analysis of these equilibrium data yielded estimates of the dissociation constant ($K_D$) for $(-)$-[3H]dihydroalprenolol binding (2.6 ± 0.28 nM by Hill plot, 2.2 ± 0.2 nM by saturation analysis) which are in good agreement with the value 4.6 nM derived from competition experiments with unlabeled alprenolol (Fig. 1 and Table IV). Scatchard (curvilinear upward) and Hill plot ($n_H < 1$) analysis of equilibrium data both suggested that site-site interactions among $\beta$-adrenergic receptors may have been retained in solubilized preparations as they exist in intact membranes (26). The retention of negatively cooperative interactions among receptor sites after solubilization has been demonstrated recently for the binding of thyrotropin-stimulating hormone to its receptor in bovine thyroid (48).

Hill coefficients less than one are also consistent with the existence of multiple binding sites with differing affinities. Steady state binding data cannot distinguish between the presence of heterogeneous sites, negatively cooperative interactions, or the simultaneous presence of both. Therefore, direct kinetic techniques similar to those applied in particulate preparations (26, 28, 49) will need to be designed and applied to confidently assess the presence of negative cooperativity among the solubilized receptor sites.

Kinetic analysis of $( -)$-[3H]dihydroalprenolol binding to solubilized sites determined at 4°C provided estimates of the rate constants of association and dissociation ($k_1 = 3.8 \times 10^4$ M$^{-1}$ min$^{-1}$, $k_2 = 2.3 \times 10^{-7}$ min$^{-1}$). The $K_D$ calculated from rate data was 0.6 nM, which is in reasonable agreement with $K_D$ values determined by other methods.

It should be noted that throughout this manuscript we have referred to estimates of the equilibrium dissociation constant ($K_D$) of $( -)$-[3H]dihydroalprenolol for the soluble $\beta$-adrenergic receptor binding sites. Since it is apparent that negatively cooperative site-site interactions may exist in these solubilized preparations, these $K_D$ values might more appropriately be described as apparent or average $K_D$ values.

Chromatography of solubilized preparations on Sepharose 6B showed that the $( -)$-[3H]dihydroalprenolol binding activity was well included in the gel ($K_{AV} \sim 0.58$ to 0.6), further verifying the soluble nature of the receptors. The estimates of molecular weight (130,000 to 150,000) were obtained in the presence of detergent and therefore should be regarded as a high estimate of the molecular weight since detergents bound to the membrane protein might increase the estimate of molecular weight (30–32).

The effect of denaturants and enzyme treatments on the binding activity of solubilized preparations suggests that the receptor sites are protein in nature. In all experiments where perturbation of binding activity was studied, whether by reagents, denaturants, or enzymes, the effects on specific binding observed were reflected always by a decrease in total binding with no change in nonspecific binding. With denaturants (urea and GuHCl) typical sharp denaturation curves were obtained, although denaturation was not assessed by other means than loss of $( -)$-[3H]dihydroalprenolol binding activity. Enzymic treatment studies showed that high concentrations of enzymes were required to affect binding activity. As suggested, this relative insensitivity might be explained by a coating of the binding sites with the detergent. The fact that phospholipase A in addition to trypsin affected the binding suggests that lipids may be functionally required for optimal binding activity.

We have demonstrated in this paper that solubilization by digitiorn treatment of the $\beta$-adrenergic receptor binding sites in a purified membrane fraction of frog erythrocytes can be achieved with maintenance of all of the essential characteristics expected of the $\beta$-adrenergic receptors. Such soluble preparations should be of great value in studies aimed at the ultimate purification of the $\beta$-adrenergic receptors as well as for reconstitution experiments directed at elucidating the relationship of the receptors to adenylate cyclase.

Acknowledgments—The electron microscopic studies in consultation were performed by Dr. J. R. Sommer, Director, Veterans Administration EM Laboratory, Veterans Administration Hospital, Durham, N.C. We are grateful to Dr. P. Jeffs, Department of Chemistry, Duke University, for assistance in interpretation of NMR and mass spectoscopic studies and to the Analytical Chemistry Department of New England Nuclear Co. for assistance in development of chromatographic procedures for alprenolol and dihydroalprenolol.

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M G Caron and R J Lefkowitz


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