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CHARACTERIZATION OF THE MAMMALIAN BETA-2 RECEPTOR IN 8 M UREA

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SUMMARY. Treatment of canine pulmonary membranes with detergent-free 8 M urea yielded a single class of large MW high-affinity binding sites exhibiting the stereospecificity and order of potency for beta-ligands characteristic of the beta receptor. This urea-solubilized receptor, demonstrating a 3-4 fold decreased affinity for beta-ligands as compared to membrane-bound and digitonin-solubilized receptor, exhibited a marked loss of activity when concomitantly exposed to solubilizing concentrations of detergent.

The release of intrinsic membrane proteins from their lipid matrix usually requires solubilization with detergents which engage these proteins in a micellar structure (1,2). Non-denaturing detergents are capable of disrupting hydrophobic interactions between proteins and lipid. Others have demonstrated, furthermore, that certain intrinsic membrane proteins can retain residual secondary structure in concentrations of urea and guanidinium salts capable of denaturing globular proteins (3). In the following report, we will document the stability of the beta-receptor to the denaturant urea and characterize the properties of the urea-solubilized receptor.

METHODS. Canine lung membranes were prepared by differential centrifugation and frozen in liquid nitrogen in 100 mM Tris, 5 mM EDTA, and 1 mM $MgCl_2$ (buffer B). For the particulate receptor assay, 100 ul membranes, 25 ul [^{3}H]-dihydroalprenolol ([^{3}H]DHA), and 25 ul buffer or competing ligand were incubated at 22^{o}C for 30 minutes, and bound-from free-ligand was separated by vacuum filtration. For the soluble receptor assay, 400 ul solubilized receptor, 50 ul [^{3}H]DHA and 50 ul buffer or competing ligand were incubated for 1 hr at 22^{o}C and then placed on ice; the receptor was then precipitated by addition of 1 ml bovine gamma globulin, 1 mg/ml, and 1 ml 25% ice-cold polyethylene glycol. The mixture was collected on GF/C filters and washed with buffer.

Urea solutions were prepared in distilled water, deionized over a mixed bed Amberlite resin, then combined with appropriate amounts of Tris, EDTA, and $MgCl_2$ to give final concentrations equal to those in buffer B. Urea solubilization was carried out by homogenization of lung membrane in urea followed by centrifugation for 2 hrs at 100,000 x g. Digitonin solutions were obtained by heating suspensions in buffer B to 90-95^{o}C. Detergent solubilization was carried out in 0.3% digitonin (Fischer) at a detergent to protein ratio of 1.5:1. The membranes were incubated for 1 hr at 22^{o}C in this solution and then centrifuged at 100,000 x g at 4^{o}C for 1 hr.

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Table 1

Membrane Protein and Receptor Solubilization With 8 M Urea or 0.3% Digitonin

	Volume (ml)	Total Protein (mg)	Protein Solubilized (%)	Total Receptor (fmol)	Receptor Solubilized (%)
Starting Membrane Preparation	10	22	———	2332	———
Digitonin Solubilization	15	7.2	33	1545	66
Urea Solubilization	13	7.8	35	1781	76

Gel filtration chromatography in Sepharose 4B-Cl (Pharmacia) was carried out in buffer B- 8 M urea on a column (1 m x 2.5 cm) run at a constant pressure head of 500 cm and a flow rate of 50 mls per hour. Protein standards were run simultaneously.

Protein concentrations were determined by the method of Lowry et al (5). Saturation binding studies were analyzed by the method of Scatchard (6). Affinity constants for unlabelled ligands were calculated from competitive inhibition studies by the method of Cheng and Prusoff (7).

RESULTS AND DISCUSSION. The beta-receptor was directly solubilized from lung membranes with buffer B-urea in concentrations of 1-8 M. Although all receptor activity was precipitated during 1 hr of ultracentrifugation in those preparations solubilized in less than 4 M urea, solubilization with urea in concentrations above 5-6 M resulted in large amounts of receptor binding in the supernatant that could be detected even after 24 hrs of ultracentrifugation at 100,000 x g. By extracting a constant amount of membrane protein with increasing volumes of 8 M urea-buffer B, a relatively constant number of specific [^{3}H]DHA binding sites were released when the protein to urea ratio was at least 1.5: 1 (w/v).

Table 1 shows that under optimal conditions a similar amount of protein is released by both the urea and digitonin protocols, with urea solubilization being slightly more effective in extracting membrane-bound receptor. The specific activity of the urea-solubilized preparation was 228 fmol/mg solubilized protein (81 fmol were released per mg of membrane protein) as compared to an initial receptor concentration in the membrane of 106 fmol/mg.

We next investigated the stability of the receptor when exposed to both digitonin and high concentrations of urea. When digitonin-solubilized receptor was dialyzed in 0.1% detergent solutions containing up to 3.6 M urea no loss of

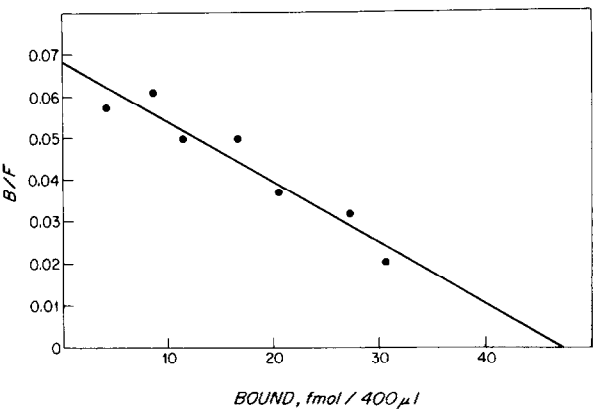
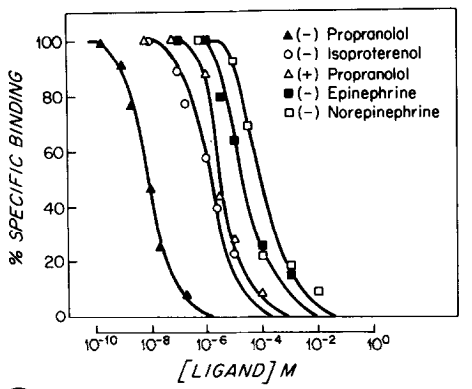


Figure 1. Scatchard plot of saturation binding curves utilizing [^{3}H]DHA (1-15 nM). Nonspecific binding was determined in the presence of 1 µM (-) propranolol. Solubilized receptor preparations were prepared by treatment of pulmonary membranes with 8M urea-buffer B followed by ultracentrifugation at 100,000 x g for 2 hrs. at 22°C. Specific binding accounted for 90% of total binding. Receptor concentration was 172 fmol per mg with a K_D of 6.9 nM.

activity was found. However, an 85% loss of specific [^{3}H]DHA binding activity resulted when the digitonin-solubilized receptor was dialyzed in digitonin solutions containing urea at concentrations above 4 M. This binding was not recovered following removal of the urea by dialysis. Furthermore, solubilization of receptor in identical volumes of buffer containing either 8 M urea, 8 M urea-0.1% digitonin, or 8 M urea-0.3% digitonin yielded significantly less [^{3}H]DHA binding in the supernatant when both detergent and 8 M urea were present. Solubilization at the highest digitonin (0.3%) concentration exhibited only 13% of the activity detected in the solubilization carried out with 8 M urea alone. Intermediate results were obtained with the 8 M urea-0.1% digitonin solubilization, in which 36% of the activity present in the 8 M urea solubilization was detected. Similarly, the activity of an 8 M urea-solubilized receptor preparation was decreased following dialysis in 8 M urea-buffer B containing 0.1% digitonin. This activity could not be recovered following removal of detergent by extensive dialysis.

The binding properties of the urea-solubilized receptor are shown in Figures 1 and 2, both by saturation binding analysis and by competitive inhibition curves



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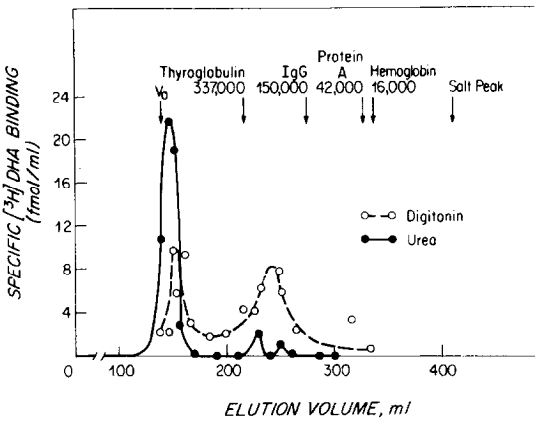

(3)

Figure 2. Competitive inhibition curves of {^{3}H}DHA (4 nM) binding to urea-solubilized receptor prepared as described in Figure 1.

Figure 3. Gel filtration chromatography of either urea-solubilized or 0.3% digitonin-solubilized receptor was carried out at 22°C utilizing Sepharose 4B-Cl equilibrated in 7.2 M urea detergent-free buffer B as described in "Methods". 4.0 ml fractions were collected. The elution position of the protein standards is indicated. These patterns are typical of 3 separate experiments, performed with both receptor preparations.

utilizing a variety of beta-ligands. Scatchard analysis of the urea-solubilized receptor indicated a K_D of 6.3 ± 0.5 nM (n=3), as compared to a typical K_D for [^{3}H]DHA in membranes of 1.3 ± 0.3 nM. The competition curves indicated a spectrum of affinities and an order of potency characteristic of the beta-2 receptor, but the calculated K_D for each ligand is approximately 3-fold greater than that determined for the membrane receptor or for the digitonin-solubilized receptor.

The receptor was further characterized by gel exclusion chromatography. When solubilized and chromatographed in 8 M urea (Fig. 3), the receptor consistently eluted in the void volume. However, if first solubilized in digitonin and then eluted under identical conditions in an 8 M urea-digitonin-free Tris buffer, receptor activity was consistently detected within the included volume, exhibiting an apparent molecular weight of 220,000-240,000 daltons. Loss of activity, however, did occur under these conditions.

Certain intrinsic membrane proteins such as lipophilin (8) are resistant to denaturation by a variety of agents, a property apparently related to the stabilization of their structure by strong hydrophobic interactions. Our findings with the beta-receptor suggest a similar role for hydrophobic interactions (3,4) in maintaining the integrity of this protein's binding site. The 3-4 fold decrease in affinity of the urea-solubilized receptor for a variety of beta-ligands indicates, however, that some perturbation of the binding site structure occurs at high concentrations.

A 70-90% loss of ligand binding effected by 8 M urea treatment of digitonin-solubilized receptor (9) and a similar decrease in ligand binding detected when the urea-solubilized receptor is dialyzed in digitonin solutions would suggest an inactivation of urea-solubilized receptor by digitonin, and also would explain the apparently decreased receptor extraction obtained in solubilization with both 8 M urea and digitonin. It is possible that the receptor, when partially unfolded in 8 M urea, is more susceptible to loss of critical phospholipid constituents during concomitant detergent treatment or that the detergent micelle hinders ligand accessibility to the receptor's binding site.

The physical state of the receptor in 8 M urea remains unclear. The urea-solubilized receptor, eluting in the void volume of an 8 M urea-detergent free gel exclusion chromatography column, demonstrates a Stokes radius considerably larger than the digitonin-solubilized receptor, which under identical conditions elutes in the included volume. It is apparent that the greater density (1.095 g/ml) of a 7.2 M urea solution may be playing a role in preventing the sedimentation of such a hydrophobic molecule, particularly if in association with membrane lipid. Failure to sediment the receptor in 7.2 M urea, however, was not merely secondary to the increased density of this solution, but rather to its disruptive effect on membrane structure: no appreciable receptor activity remained in the supernatant after lung membrane in a 30% sucrose solution, which has a greater density than 7.2 M urea, was ultracentrifuged at 100,000 x g for 1 hr.

These findings suggest that urea extraction may provide an approach for obtaining receptor in a state such that lipid factors typically exchanged during

detergent extraction (1,2,10) remain associated with the receptor protein. Such a preparation would provide an alternative to the use of detergent-solubilized receptor in attempts to reconstitute receptor function in artificial lipid membrane bilayers. Furthermore, if other intrinsic membrane proteins also remain associated with the urea-extracted receptor, then reconstitution assays may provide some clue as to those constituents which are in proximity to or are functionally associated with the receptor in the membrane.

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