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SEPARATION OF SOLUBILIZED ALPHA AND BETA ADRENERGIC RECEPTORS BY AFFINITY CHROMATOGRAPHY

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SUMMARY

Hepatic plasma membrane alpha and beta-adrenergic receptors have been solubilized in active form by digitonin. The soluble receptors were identified by the binding of [^{3}H]WB4101 and [^{3}H]dihydroalprenolol respectively, and were characterized by competition studies with adrenergic agents. Affinity chromatography was used to separate the solubilized receptors by selective adsorption of beta-adrenergic receptors to an alprenolol-substituted agarose gel. The findings document that the active alpha and beta-adrenergic binding sites do not simultaneously reside on the same macromolecule.

INTRODUCTION

Ahlquist first suggested that the physiologic effects of catecholamines were mediated by two distinct forms of adrenergic receptors, which he termed alpha and beta (1). Much interest has focused, in recent years, on the possible relationships of these two receptor types (2). It has not been clear whether the two receptors are separate entities, interconvertible, or different domains of the same macromolecule. The advent of radioligand binding techniques for studying these receptors has made possible a direct approach to such questions in biochemical terms. Although beta-adrenergic receptors have been solubilized and even partially purified from amphibian and avian erythrocytes (3-5) to date there have been no reports of solubilization of the alpha-adrenergic receptors.

Both alpha and beta-adrenergic receptors have previously been detected in rat liver by at least two different lines of investigation: Exton and co-workers have demonstrated alpha and beta-adrenergic effects on glycogenolysis, glucose output, and adenosine 3',5'-monophosphate formation (6), and several laboratories have studied membrane binding of the alpha-adrenergic antagonist (^{3}H)dihydroergocryptine (7,8) and the beta-adrenergic antagonists (^{125}I)iodohydroxybenzylpindolol (9) and (^{3}H)dihydroalprenolol (7). In this communication, we report the solubilization in digitonin of the hepatic alpha-adrenergic receptor in active form and the characterization of its binding properties. We also report

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the solubilization of the hepatic beta receptor and the separation of solubilized alpha and beta-adrenergic receptors using selective adsorption of beta receptors by an affinity support consisting of a conjugate of the potent beta-adrenergic antagonist alprenolol covalently linked to Sepharose 6B.

MATERIALS AND METHODS

(^{3}H)Dihydroalprenolol (DHA), a potent beta-adrenergic antagonist (48.6 Ci/mmol) and (^{3}H)WB4101 (25 Ci/mmol), a potent alpha-adrenergic antagonist, were from New England Nuclear. Alprenolol affinity gel was prepared as previously described (4). Sources of other materials have been previously described (3,8). Female Wistar rats, 100 to 125 grams, were adrenalectomized (by Charles River) and given isotonic saline drinking water for six to eight days prior to killing by decapitation. Adrenalectomy has been found to increase the number of hepatic beta-adrenergic receptors (7,9). Hepatic plasma membranes were prepared by an adaptation of the method of Neville(10), as previously described (8), and were stored at -70°C until used. Protein concentrations were determined by the method of Lowry (11) using bovine serum albumin as a standard.

Particulate alpha-adrenergic receptor binding was assayed on membranes pre-incubated for 10 minutes at 25°C with 50 μM pargyline exactly according to Clarke et. al (8), except that the radioligand (^{3}H)WB4101 (0.4 to 0.6 nM) was used instead of (^{3}H)dihydroergocryptine, and sodium ascorbate (0.016%) and catechol (0.011%) were included in the incubation. "Specific" receptor binding was defined as that which was competed for by 10 μM phentolamine, or 1 mM (-)epinephrine, and amounted to 65 to 75% of total binding. All results refer to "specific"binding.

Particulate beta-adrenergic receptor binding was assayed according to Guellaen et al. (7). Liver membranes containing 300 to 800 μg protein were incubated with 2 nM (^{3}H)DHA and 10^{-4}M phentolamine. Specific binding (displaceable by 10 μM (-)isoproterenol)represented 50-70% of total binding.

Solubilization - Liver membranes, 2 to 4 mg/ml protein, were solubilized with 1% digitonin in 50 mM sodium phosphate buffer, pH 7.4, in the manner previously described for the frog erythrocyte (3).

Soluble alpha receptors - Membranes previously incubated for 10 minutes at 25°C with 50 μM pargyline were solubilized as above. Soluble preparation containing about 600 μg protein was added to 1 to 2 nM (^{3}H)WB4101, a competing adrenergic agent, 0.016% sodium ascorbate, and 0.011% catechol in a total volume of 600 μl. Samples were incubated 90 minutes at 4°C. Bound radioactivity was determined as previously described (3). Specific binding (displaceable by 1 mM (-)epinephrine or 10 μM phentolamine) represented about 70% of the total binding to protein.

Soluble beta receptors were assayed as above, except that the radioligand was (^{3}H)DHA, 3 nM, and 10^{-4}M phentolamine was present. Specific binding (displaceable by 10 μM (-)isoproterenol) represented 50% of total binding to protein.

Affinity chromatography of solubilized hepatic membranes - A column containing 1 to 2 ml of Sepharose 6B alprenolol gel, equilibrated with 0.2% digitonin in 50 mM phosphate buffer pH 7.4, was loaded with 3 ml of 1% digitonin-solubilized hepatic membrane preparation at 23°. The column effluent was collected and the column was washed with equilibration buffer. In some experiments, after washing the column the beta-adrenergic receptor activity was eluted with 100 mM (±)isoproterenol or 1 mM (-)alprenolol in 0.2% digitonin, 50 mM phosphate buffer pH 7.4 at 23°, and the eluted samples were "desalted" by passage over Sephadex G-50 columns. Soluble alpha and beta-adrenergic receptor activities were assayed as described above. This alprenolol affinity column has been used to obtain extensive purification of the frog erythrocyte beta-adrenergic receptor (4).

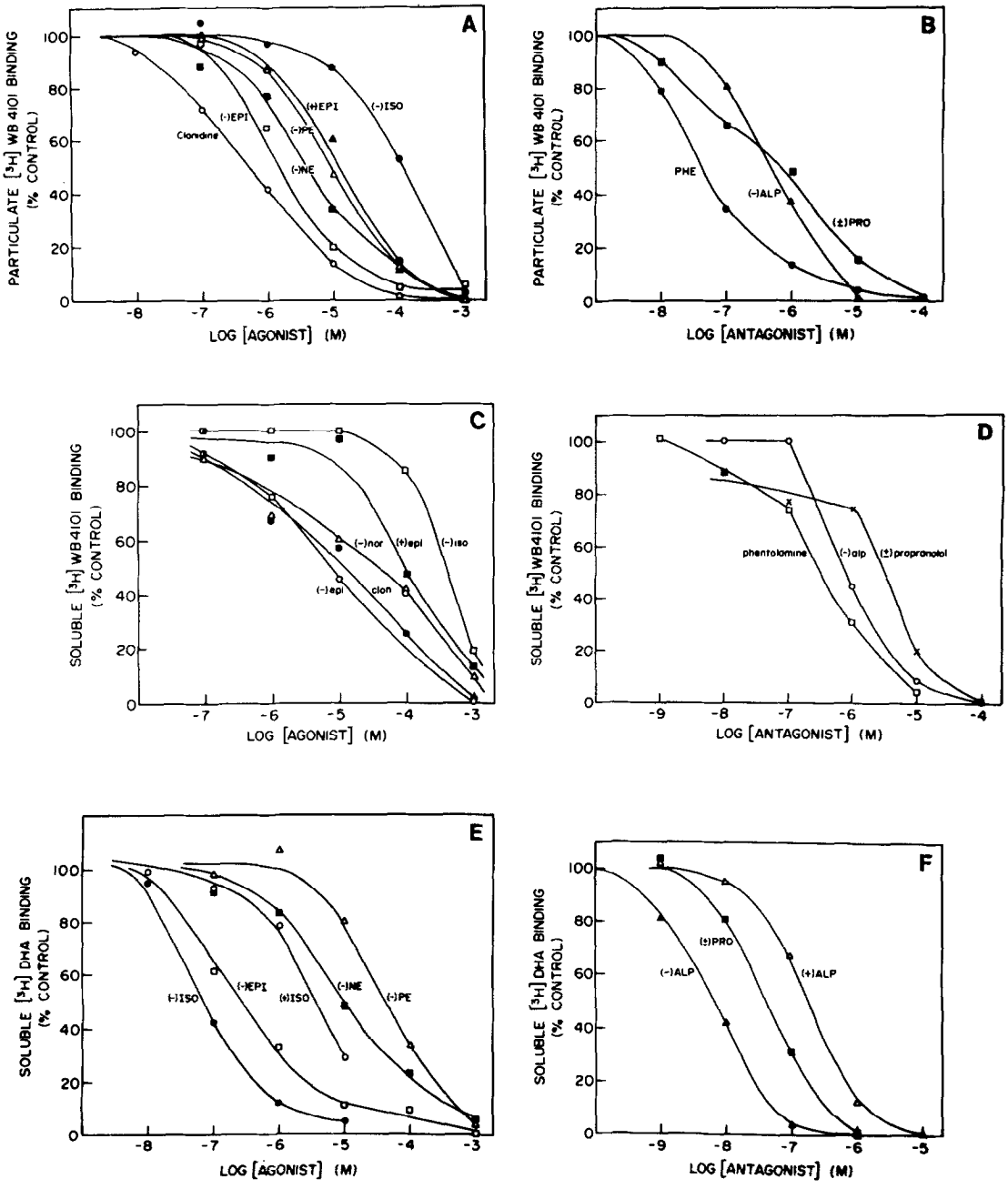

Fig. 1. Competition by adrenergic agonists and antagonists for (^{3}H)WB4101 binding to hepatic plasma membranes (A,B), and for (^{3}H)WB4101 binding (C,D) and (^{3}H)DHA binding (E,F) to soluble preparations of hepatic plasma membranes. Binding studies were performed, as described in Methods, in the absence and presence of the indicated concentration of competitor, and the percent of control specific binding was calculated for each experiment. Each point is the average of two to seven separate experiments, each performed in duplicate.

TABLE I

Dissociation Constants (K_D's) of Adrenergic Agents[a]

	Alpha		Beta	
	Particulate	Soluble	Particulate	Soluble
Agonists	μM	μM	μM	μM
(-)Epinephrine	1.0	4.0	NM	0.13
(+)Epinephrine	10	40	NM	NM
(-)Norepinephrine	2.1	15.	NM	4.8
(-)Isoproterenol	55.	90.	0.11	0.034
(+)Isoproterenol	NM	NM	12.	1.7
(-)Phenylephrine	4.5	1.	NM	22.
Clonidine	0.25	4.	NM	NM
Antagonists				
(-)Alprenolol	0.25	0.2	0.008	0.003
(+)Alprenolol	NM	NM	0.18	0.10
(±)Propranolol	0.35	0.8	NM	0.020
Phentolamine	0.021	0.08	NM	NM

[a]Dissociation constants of particulate and digitonin-solubilized alpha and beta-adrenergic receptors were calculated from displacement curves (Figure 1)by the method of Cheng and Prusoff (12). NM-not measured.

RESULTS AND DISCUSSION

Particulate alpha receptors - To investigate the usefulness of (^{3}H)WB4101 as a ligand for hepatic alpha-adrenergic receptors, saturation and competition experiments were performed on the hepatic plasma membranes. Scatchard plots of saturation data (not shown) were linear and yielded a dissociation constant (K_D) of 0.7 nM and saturation specific binding levels of 800 fmol/mg protein. Characterization of the binding site by competition studies (Fig. 1A and B) revealed a typical alpha adrenergic order of potency: (-)epinephrine> (-)norepinephrine> phenylephrine> (-)isoproterenol. Phentolamine was more potent than (-)alprenolol or (±)propranolol. It was concluded that (^{3}H)WB4101 binds to the hepatic alpha-adrenergic receptor.

Particulate beta receptors - It has already been shown that (^{3}H)DHA binds the beta receptor in hepatic plasma membranes (7). This is confirmed by the present studies. Table 1 shows the high affinities for (-)alprenolol and (-)isoproterenol, and the 20 to 100 fold stereoselectivities for the (-) over the (+)isomers. Young female adrenalectomized Wistar rats (7) were used throughout this study because such beta receptor specific, stereoselective binding of (^{3}H)DHA was not demonstrable in hepatic membranes from older (300-500 g) CD strain male rats, whether adrenalectomized or not (data not shown).

Soluble alpha receptors - Digitonin (1%, 10 mg/ml) solubilized 50% of hepatic membrane proteins. Digitonin concentrations less than 5 mg/ml yielded fewer soluble alpha receptors in a dose-dependent fashion. Competition studies for

(^{3}H)WB4101 binding to the soluble alpha receptor are shown in Figure 1,C and D, and reveal the alpha-adrenergic potency order (-)epinephrine> (-)norepinephrine> (-)isoproterenol for agonists: phentolamine> (-)alprenolol and (±)propranolol for antagonists; and a 10-fold stereoselectivity for (-)versus (+)epinephrine. Comparison of dissociation constants of particulate and soluble alpha receptors (Table 1) indicates a 2 to 8-fold decrease in affinity upon solubilization for most of the drugs studied. Saturation curves (data not shown) gave saturation levels around 40 fmoles/mg protein and half-maximal saturation levels at a WB4101 concentration of 1 nM. The numbers of alpha receptors appearing in the soluble preparation ranged from 3 to 10% of those present in the particulate preparation. Deletion of divalent cations from the sodium phosphate buffer gave higher yields. The yield was not improved by pre-binding of (^{3}H)WB4101 to the membranes before solubilization, or by solubilizing in the presence of 0.25 M sucrose, 100 mM NaCl, 2 mM sodium metabisulfite, or 0.1 mg/ml dithiothrietol. Detergents which were found ineffective in solubilizing active alpha receptors included Tritons X-100,X-305, X-405, Lubrol PX, Tween 80, octyl sodium sulfate, and a combination of digitonin and Triton X-100. The frog erythrocyte beta-adrenergic receptor, which was successfully solubilized by digitonin (3), has also been shown to be inactivated by many other detergents (3). Thus digitonin appears to be a uniquely useful detergent for both adrenergic receptors.

The radioligand (^{3}H)dihydroergocryptine, which identifies alpha-adrenergic receptors in particulate hepatic membranes (7,8) was not useful for the detection of soluble alpha receptors by the Sephadex G-50 chromatography assay because of its high partition into digitonin micelles. (^{3}H)WB4101 and (^{3}H)DHA did not display this high partition into digitonin micelles and therefore were useful for detecting soluble alpha and beta receptors.

Soluble beta receptors - The characterization of the soluble beta receptor is shown by Figure 1, E and F, and gives the typical beta-adrenergic order of potency: (-)isoproterenol> (-)epinephrine> (-)norepinephrine> (-)phenylephrine. The stereoselectivities for the (-)isomers of isoproterenol and alprenolol are also shown. Under the conditions used,about 30% of the particulate beta receptors were recovered in the soluble preparations.

The binding studies presented here show that both alpha and beta-adrenergic receptors could be detected in the digitonin-solubilized preparations. Although the solubilized receptors are numerically only a fraction of those in the starting membranes, data from the characterization studies (Table I) suggest that the soluble receptors are similar to and representative of the membrane-bound receptors. The lower recovery of alpha than beta receptors in the soluble preparations and the affinity decreases upon solubilization seen with the alpha but not the

TABLE 2

Separation of Alpha and Beta-Adrenergic Receptors Using Selective Adsorption of Beta Receptors by an Alprenolol Affinity Column[a]

Specific Binding of Soluble Preparation (fmol/mg protein)					Percent Decrease in Specific Binding in Affinity Column Effluent	
	(^{3}H)WB4101 (alpha)		(^{3}H)DHA (beta)		(^{3}H)WB4101 (alpha)	(^{3}H)DHA (beta)
Expt. #	Control	Column Effluent	Control	Column Effluent		
1.	7.1	6.7	8.	0.4	6%	95%
2.	21.	26.	28.	8.2	0%	71%
3.	15.	16.	18.	4.3	0%	76%

[a]Hepatic plasma membranes were solubilized as described in Methods and were divided in half. One aliquot was passed over an alprenolol affinity gel at 23°C as described in Methods while the control aliquot was kept at 23°C for the same length of time. Each aliquot was then assayed for soluble alpha and beta-receptor binding and for protein. Ligand concentrations were 1.6 to 2.2 nM (^{3}H)WB4101 and 1.4 to 11.0 nM (^{3}H)DHA. Protein concentrations of the affinity column effluents were decreased 7 to 29% from the starting material.

beta receptor indicate a higher lability of the alpha receptor when removed from the membrane environment.

Affinity chromatography -Passage of soluble preparations containing 1% digitonin over an alprenolol affinity column in 5 separate experiments resulted in an average 82% decrease in specific (^{3}H)DHA binding with no decrease in (^{3}H)WB4101 binding. Results of three representative experiments are shown in Table II. Thus the soluble alpha and beta receptors were physically and functionally separated by the adsorption of the beta receptors to the affinity gel while the alpha receptors were unretarded.

The possibility that the decreased (^{3}H)DHA binding in the affinity column effluent was due to the artifact of competition for soluble receptors by unlabeled alprenolol leaching off the affinity column gel was assessed by "desalting" the affinity column effluents on Sephadex G-50 columns. This"desalting" procedure has previously been shown to remove competing alprenolol (10-100 mM) from digitonin-solubilized frog erythrocyte beta-adrenergic receptor preparations (4). If leaching were present, desalting of the affinity column effluents would restore the lost (^{3}H)DHA binding activity. This was not observed, as both desalted and non-desalted affinity column effluents showed 75 to 80% decrease in (^{3}H)DHA binding activity from the starting preparation. This shows that alprenolol leaching is not a problem in using this affinity gel for solubilized liver

preparations, as has also been demonstrated previously for solubilized frog erythrocyte preparations (4).

Recovery of beta receptors from the affinity gel was investigated in three experiments. After the loading of the affinity gel by passage of the soluble preparation through the column, washing the gel with buffer containing 0.2% digitonin eluted virtually no binding activity. Further elution of the column with 100 mM isoproterenol or 1 mM (-)alprenolol in the presence of 0.2% digitonin buffer, followed by desalting of the effluent by G-50 columns and then the routine (^{3}H)DHA binding assay, recovered 17% of the adsorbed beta binding activity. No alpha receptors were detectable in these fractions. The beta receptor recovery is somewhat lower than that of the frog erythrocyte receptor (30-60%) (4), but the soluble hepatic receptor might be more labile under these conditions.

In summary, hepatic plasma membrane alpha and beta-adrenergic receptors have been solubilized in an active form, characterized, and separated by selective adsorption of beta receptors onto an alprenolol affinity gel. These studies represent the first report of the solubilization of an alpha-adrenergic receptor. They also show that the active alpha and beta binding sites detected in the soluble preparation are separable and therefore do not reside simultaneously on the same macromolecule. Such separation studies demonstrating the resolution of various components of a solubilized membrane preparation are a useful and powerful application of affinity chromatography. Digitonin-solubilized hepatic alpha-adrenergic receptors may be useful in further purification and reconstitution studies.

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