β-ADRENERGIC RECEPTORS: SOLUBILIZATION OF (-)[^{3}H]ALPRENOLOL BINDING SITES FROM FROG ERYTHROCYTE MEMBRANES

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SUMMARY: The β-adrenergic receptors ((-)[^{3}H]alprenolol binding sites) present in a purified preparation of frog erythrocyte membranes have been solubilized with digitonin and assayed by equilibrium dialysis with (-)[^{3}H]alprenolol. At a concentration of 0.5-1% the detergent solubilizes about 80% of the receptor binding activity. The soluble receptor sites are not sedimented at centrifugal forces up to 105,000 xg for two hours, pass freely through Millipore filters of 0.22 μ pore size and fractionate on Sepharose 6B gel with an apparent molecular weight of 130-150,000 in the presence of digitonin. The soluble receptor sites retain all of the binding characteristics of the membrane-bound receptors. β-adrenergic agonists and antagonists compete with (-)[^{3}H]alprenolol for occupancy of the soluble sites with affinities which are directly related to their β-adrenergic potency on membrane-bound adenylate cyclase.

Those physiological actions of catecholamines which result in increased intracellular levels of cyclic AMP are mediated by β-adrenergic receptors (1). In most tissues β-adrenergic receptors are coupled to the enzyme adenylate cyclase (2). The use of potent competitive β-adrenergic antagonists labelled to high specific activity has recently permitted the identification of these receptors by direct binding techniques. Binding sites with the specificity of the β-adrenergic receptors have been described in avian (3-6) and amphibian (7-10) erythrocytes as well as mammalian myocardium (11),brain (12) adipose tissue (13) and human leukocytes (14). The β-adrenergic receptor sites of frog erythrocytes, identified with (-)[^{3}H]alprenolol possess all the characteristics expected of the physiological β-adrenergic receptors (8-10). The binding is rapid, reversible and saturable. The sites show high affinity for β-adrenergic agonists and antagonists and no affinity for β-adrenergic agents. The interaction of β-adrenergic agonists and antagonists with the binding sites displays marked stereo-specificity. The (-)isomers of these agents have affinities which are 1 to 2 orders of magnitude greater than the (+)isomers. Non-physiologically active catechol compounds do not interact with the membrane-bound sites (7-10). In this communication we report the solubilization of

these (-)[^{3}H]alprenolol binding sites from an erythrocyte membrane fraction by treatment with digitonin. The solubilized binding sites retain all the essential properties (described above) which indicated the identity of the membrane-bound (-)[^{3}H]alprenolol binding sites with the β-adrenergic receptors.

MATERIALS AND METHODS

(-) and (+) alprenolol hydrochloride were gifts of Dr. Ablad (Hassle Pharmaceuticals) and (-) and (+) propranolol were from Ayerst Pharmaceuticals. (+)Isomers of isoproterenol, epinephrine and norepinephrine bitartrate were gifts from Sterling Winthrop Pharmaceuticals. (-)Isomers of isoproterenol, epinephrine and norepinephrine bitartrate were from Sigma as were all other compounds used. Digitonin was obtained from Sigma and Fisher. (-)Alprenolol hydrochloride was tritiated to a specific activity of 17-33 Ci/mmol at New England Nuclear Co. by catalytic reduction with tritium using paladium as the catalyst. We have recently determined by mass spectroscopy that as expected the structure of (-)[^{3}H]alprenolol is that of dihydroalprenolol. The radiolabeled material has biological activity identical to that of native (-)alprenolol. Sources for other materials were as described previously (8).

Membrane Preparations: Preparation of a membrane fraction from erythrocytes of frogs (Rana Pipiens) was as described earlier (9). In brief, lysates of red cells were washed free of hemoglobin and the pelleted material was fractionated by centrifugation through 50% sucrose buffer. The material which did not sediment through sucrose was pelleted, washed and resuspended in 50 mM Tris-HCl pH 7.4 buffer. Membranes prepared by this method showed a 4.3 fold increase in the specific activity of basal adenylate cyclase and a 9.5 fold enrichment of (-)[^{3}H]alprenolol binding over unfractionated lysates.

(-)[^{3}H]Alprenolol Binding Assays: Assays of (-)[^{3}H]alprenolol binding in membrane fractions were performed by a centrifugal technique as previously described (8). (-)[^{3}H]alprenolol binding to solubilized preparations was assayed by equilibrium dialysis.

Proteins: Protein determinations were performed according to the method of Lowry (15). Estimations of samples containing digitonin were corrected for the presence of digitonin.

RESULTS AND DISCUSSION

Solubilization of (-)[^{3}H]alprenolol binding sites. Treatment of a purified frog erythrocyte membrane fraction with the plant glycoside digitonin released the (-)[^{3}H]alprenolol binding sites in a solubilized

form. Table I summarizes the results obtained when membranes were treated with digitonin. Concentrations of 0.5% and 1.0% were equally effective in extracting the membrane binding sites. Treatment of erythrocyte membranes with digitonin appeared to be quite specific in releasing the binding sites in a solubilized active form. Treatment under similar conditions with various other detergents did not solubilize the binding sites in an active form. The detergents and concentrations tested were: Lubrol PX 0.25, 1.0%; Lubrol WX, 1%; Tritons X-100, 1%; X-305, 1%; N-101, 1%;

Table I. Solubilization of (-)[^{3}H]alprenolol binding sites from purified frog erythrocyte membranes.

Preparation	Protein	(-)[^{3}H]Alprenolol Bound		
	μ g/ml	pmoles/ml	pmoles/mg	yield % Binding Sites Solubilized
Purified Membrane Fraction	1700 ± 184	2.47 ± 0.37	1.45 ± 0.21	———
Solubilized Preparations				
0.5% Digitonin	561 ± 39.8	2.02 ± 0.20	3.60 ± 0.36	87.8 ± 22.0
1.0% Digitonin	846 ± 66.3	2.09 ± 0.20	2.47 ± 0.24	89.6 ± 19.6

The membrane preparations were obtained as described in Methods section (9). Membrane protein (4.5-5.5 mg) was suspended in 3 ml of 50 mM Tris HCl pH 7.4 at 4° in buffer containing the indicated concentrations of digitonin. The suspensions were allowed to stand 30-60 min at 0° and then were centrifuged at 30,000 xg for 30-45 min. The supernatants constituted the solubilized preparations on which (-)[^{3}H]alprenolol binding assays were performed. Samples of 500-700 μ g of solubilized protein were dialyzed in the presence or absence of 10 μ M (±)propranolol against 1% digitonin solutions containing 50 mM Tris HCl, pH 7.4 and 10 nM (-)[^{3}H]alprenolol in a total volume of 2 ml. After 16-20 hours of incubation quadruplicate samples were removed from both sides and counted. Specific binding was determined as the difference between total binding and that observed in the presence of 10 μ M (±)propranolol or (-)alprenolol (nonspecific binding). Non-specific binding in the presence of 1% digitonin varied from 20-45% of total binding. Results shown are means of quadruplicate determinations from 3 experiments ± SE.

CF-54, 1%; sodium deoxycholate, 0.25, 1%; octylsodium sulfate 0.1, 0.25, 1%; lithium diiodosalysilate 0.3 M, 0.15 M. Lysolecithin, phospholipase A and EDTA were also tested and found ineffective.

The soluble state of the digitonin extracted membrane preparations was verified by several independent experimental techniques. Thus the (-)[^{3}H]alprenolol binding activity was not sedimented by centrifugation at 105,000 xg for 2 hours; it passed through Millipore filters of 0.22 μ pore size. The soluble binding sites chromatographed on Sepharose 6B with an apparent molecular weight of 130-150,000 in the presence of the detergent as compared with several proteins of known molecular weight (thyroglobulin, apoferritin, γ-globulin, transferrin, ovalbumin). The soluble receptors chromatographed as a single major peak with a small amount of binding activity in the void volume. Electron microscopy of these preparations revealed no evidence of membrane structure[1].

(-)[^{3}H]alprenolol binding to these solubilized preparations as assayed by equilibrium dialysis was linearly related to the concentration of protein up to 0.7 mg/ml.

Stereospecific Interaction of β-adrenergic Agonists and Antagonists with the Soluble (-)[^{3}H]alprenolol binding Sites. Fig. 1 demonstrates the ability of several β-adrenergic agonists to compete with (-)[^{3}H]

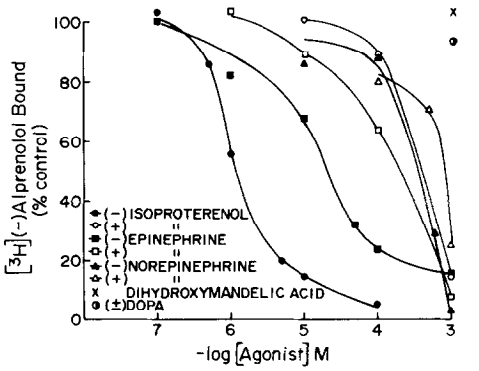

Fig. 1 Inhibition of (-)[^{3}H]alprenolol binding to solubilized preparations by β-adrenergic agonists and other agents. (-)[^{3}H]alprenolol binding was assessed by equilibrium dialysis in the absence and in the presence of increasing concentrations of β-adrenergic agents. (±)DOPA: ± dihydroxyphenylalanine and dihydroxymandelic acid were tested at 1 mM. Specific binding was determined as indicated in the legend of Table I. 100% or control specific binding was 1.42 ± 0.04 pmol/mg. The results shown are the means of quadruplicate determinations from 2 experiments.

[1]Caron, M.G. and Lefkowitz, R.J. Manuscript in preparation.

alprenolol for binding to the solubilized sites. These data indicate that the interaction of β-adrenergic agonists with the soluble binding sites displays marked stereospecificity. The (-)isomers of isoproterenol, epinephrine and norepinephrine are much more potent than their respective (+)isomers. The potency series of these agonists for inhibition of binding ((-)isoproterenol> (-)epinephrine>> (-)norepinephrine) is typical of the β-adrenergic receptor. Physiologically inert catechol compounds, e.g. dihydroxymandelic acid and (±)dihydroxyphenylalanine at concentrations of 100 μM did not interact with the sites. The ability of all three agonists ((-) and (+)isomers) to inhibit (-)[^{3}H]alprenolol binding

Table II. Concentrations of β-adrenergic agonists and antagonists causing half-maximal inhibition of (-)[^{3}H]alprenolol binding to membrane-bound and solubilized β-adrenergic binding sites.

Compounds	Concentration for half-maximal inhibition of (-)[^{3}H]alprenolol binding μM	
	Membrane - Bound Receptors	Solubilized Receptors
(-)Alprenolol	0.015	0.017
(-)Propranolol	0.023	0.015
(+)Alprenolol	0.8	1.0
(+)Propranolol	1.5	2.2
(-)Isoproterenol	2.0	1.1
(-)Epinephrine	20	20
(-)Norepinephrine	250	330
(+)Isoproterenol	800	400
(+)Epinephrine	600	200
(+)Norepinephrine	1000	800

The values shown were determined by performing competition curves for each agonist and antagonist. Binding was measured as described in the methods section, by a centrifugation assay for the membrane-bound sites and equilibrium dialysis for the solubilized preparations (7-8).

in solubilized preparations correlates well with their ability to do so in membrane-bound preparations (7-8). Table II presents a comparison of both sets of data.

β-adrenergic antagonists interact with the (-)[^{3}H]alprenolol binding sites with high affinity and stereospecificity. Data shown in Fig. 2 indicate that (-)alprenolol and (-)propranolol are equally potent in competing with (-)[^{3}H]alprenolol for the binding sites. (-)Isomers of alprenolol and propranolol are 2 orders of magnitude more potent than the (+)isomers of these compounds suggesting that the solubilized binding sites retain the same strict stereospecific binding properties apparent with the membrane-bound receptor sites (8,10). The solubilized binding sites show no affinity for the α-adrenergic antagonist phentolamine. Table II demonstrates close agreement between the concentrations of β-adrenergic agents ((-) and (+)isomers) causing 1/2 maximal inhibition of (-)[^{3}H]alprenolol binding to the soluble sites and to the membrane-bound sites.

In addition binding of (-)[^{3}H]alprenolol to these soluble receptor sites is rapid and reversible as assessed by a Sephadex G-50 filtration assay[1].

The solubilized (-)[^{3}H]alprenolol binding sites described in this study appear to possess all of the characteristics expected of the physiological β-adrenergic receptor binding sites. The sites possess

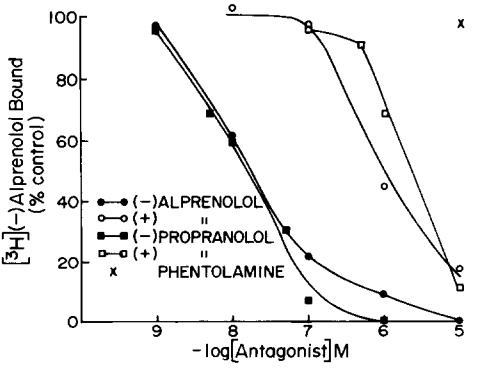

Fig.2 Inhibition of (-)[^{3}H]alprenolol binding to solubilized erythrocyte membrane preparations by adrenergic antagonists. (-)[^{3}H]alprenolol binding competition curves were performed as described in the legend of Fig. 1. Phentolamine was tested at 10 μM. 100% or control specific binding was 2.46 ± 0.18 pmol/mg. The results shown are the mean of quadruplicate determinations from 2 experiments.

high affinity for β-adrenergic antagonists and no affinity for the α-adrenergic antagonist phentolamine. The affinity of the sites for β-adrenergic agonists is also exactly as would be expected from physiological and biochemical data (isoproterenol> epinephrine>> norepinephrine). Moreover, for both agonists and antagonists these sites exhibit strict stereospecificity. The (-)isomers of β-adrenergic agonists and antagonists are much more potent than the (+)isomers.

These findings indicate that treatment of the erythrocyte membrane preparations with digitonin releases the β-adrenergic receptor binding sites in functional form with full retention of all the binding properties that can be observed in intact tissue or membrane-bound preparations (8). The successful solubilization of β-adrenergic receptors with unaltered binding characteristics reported here should now permit serious attempts at purification and physicochemical characterization of the receptor structures. In addition, it provides a system in which the nature of the interactions between the soluble β-receptors and soluble adenylate cyclase can be studied.

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