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A Competitive Receptor Binding Radioassay for β-1 and β-2 Adrenergic Agents (42522)

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Abstract. A rapid and sensitive competitive receptor binding assay for β-1 and β-2 adrenergic binding for adrenergic agents has been developed. The steps that are critical for the success of the assay are given in detail so that the assay can be set up in any routine laboratory with relative ease. The rationale behind the use of specific reagents is discussed. The assay requires microgram quantities of test compound, a radiolabeled specific β adrenergic antagonist [^{3}H]dihydroalprenolol (DHA), and turkey erythrocyte β-1 and rat erythrocyte β-2 receptor membranes. Serial dilutions of sample are incubated with appropriate receptor membranes and DHA for 1 hr at room temperature. After equilibrium is attained, the bound radioligand is separated by rapid filtration under vacuum through Whatman GF/B filters. The amount of bound DHA trapped on the filter is inversely proportional to the degree of β-1 or β-2 adrenergic binding of the sample. Separation of bound from free radioligand by filtration permits rapid determination of a large number of samples. This assay quantitates and differentiates β-1 and β-2 adrenergic binding of synthetic adrenergic agents.

β Adrenergic receptors have been classified into two subtypes based on differences in the pharmacological specificity of receptor-mediated responses (1). β-1 adrenergic receptors have approximately the same affinity for epinephrine and norepinephrine and have been implicated in the regulation of lipolysis (2) and cardiac chronotropic and inotropic responses (1, 3, 4). β-2 adrenergic receptors have a higher affinity for epinephrine than for norepinephrine and are involved in smooth muscle relaxation in the lung, uterus, and the peripheral vascular bed (1) as well as in the regulation of glycogenolysis in skeletal muscle and liver (5). Jolly *et al.* demonstrated that the β-2 receptor was the critical receptor involved in fat cell lipolysis in rats (6).

The β-2 agonist clenbuterol has been shown to induce partition of energy flow between fat and protein synthesis (7). Furthermore, catecholamines have been found to induce a time-dependent and dose-dependent stimulation of amino acid transport across the cell membrane and incorporation into protein (8).

The radioligand binding technique is commonly used in receptor research. For example, it is used for characterization of the pharmacological binding properties of receptors in intact cells, isolated membranes, and soluble preparations; in tagging the receptor through purification procedures (9); in studying regulation of the number of the receptors in tissues under a variety of physiological and pathophysiological circumstances (9); in comparing the pharmacology of the mechanisms of receptor–effector coupling (10); and in studying β-1 and β-2 adrenergic subtypes (9, 10). Despite aforementioned applications, the radioactive ligand binding procedure has never been applied for screening of experimental compounds with respect to their adrenergic activities. We have been successful in employing this concept as a screening for β-1 and β-2 adrenergic activities of synthetic compounds.

Materials and Methods. The radioactive ligand [^{3}H]dihydroalprenolol (DHA) and the scintillation cocktail Aquasol were purchased from New England Nuclear Corp., dimethyl sulfoxide (DMSO) was supplied by J. T. Baker Co., and all other chemicals were obtained from Sigma Chemical Co.

Preparation of β adrenergic receptor membrane. β-1 and β-2 adrenergic receptor membranes were isolated from turkey and rat erythrocytes, respectively (11–13). Freshly drawn heparinized whole blood from turkeys or rats was centrifuged for 5 min at 500*g*. The plasma was withdrawn and the remaining erythrocytes were suspended in 145 m*M* sodium chloride (NaCl) solution and centrifuged as above. The cells were resuspended and centrifuged twice more. The cells were hemolyzed in 10 vol of cold distilled water containing 2 m*M* dithiothreitol (DTT), 100 μ*M* phenyl-

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methylsulfonyl fluoride, 5 μg/ml leupeptin, 20 μg/ml bacitracin, 0.1% bovine serum albumin (BSA), and 1 unit/ml aprotinin, and centrifuged at 30,000*g* for 5 min. The bottom gelatin layer was discarded. The top layer was centrifuged five times at 30,000*g* with the pellet being resuspended in the buffer (145 m*M* NaCl, 1 m*M* ethylene glycol bis(β-aminoethyl ether)*N,N,N,*tetraacetic acid (EGTA), 2 m*M* magnesium chloride ($MgCl_2$), 10 m*M* Tris, and 10% glycerol, pH 7.4). The resuspended membranes were further purified with differential centrifugation at 2000*g* for 10 min by using a sucrose cushion (saturated sucrose solution) and subsequently stored in small aliquots at −70°C. The protein concentration of receptor membranes was determined by the biuret method.

Assay procedure. To duplicate incubation tubes, 160 μl suspended membranes (200 μg protein), 20 μl of compound in solution or buffer, and 20 μl of [^{3}H]DHA (98.5 Ci/mmole) 0.25 μCi were added. Tubes were incubated at room temperature for 1 hr and then filtered rapidly under vacuum through siliconized Whatman GF/B filters using a Brandel M-48R cell harvester. The filters were rinsed three times with 5 ml of buffer (145 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), and 10 m*M* Tris, pH 7.4) and subsequently counted in 10 ml of Aquasol by liquid scintillation counter. Specific binding of [^{3}H]DHA

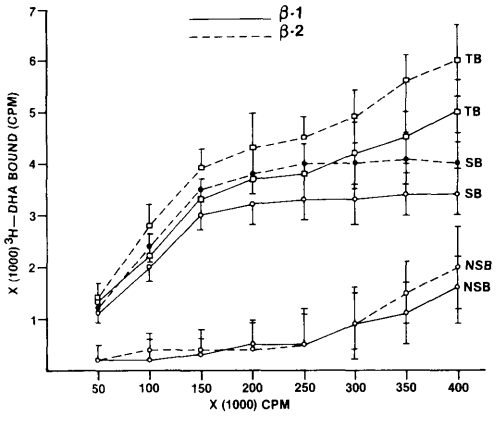

FIG. 1. Saturation curve β-1 and β-2 receptor membranes. [^{3}H]Dihydroalprenolol (DHA) values are the means ± SEM of five experiments performed in duplicate. TB, total binding; NSB, nonspecific binding; SB, specific binding. Total cpm = 200,000.

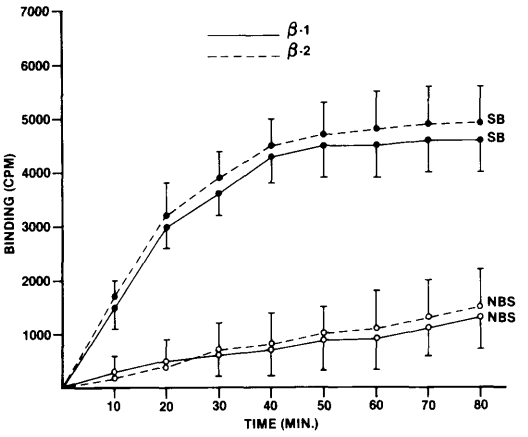

FIG. 2. Time vs. binding. Values are the means ± SEM of five experiments performed in duplicate. SB, specific binding; NSB, nonspecific binding.

was defined as the excess over blank containing 100 μ*M* nonradioactive DHA or isoproterenol. Serial dilutions of each compound were tested and Cimaterol (CL 263,780) was used as a reference compound.

Results. A variety of conditions were examined for the binding procedure. Several buffers (including Krebs, Ringers, Hepes, phosphate, and Tris) were tested at various pH levels. Binding was maximum in Tris buffer with 10% glycerol (10 m*M*, pH 7.4); 10 n*M* DHA was found to be the optimum concentration for binding (Fig. 1). With the higher concentrations, nonspecific binding increased substantially (Fig. 1). Studies of the time dependence of the binding showed that receptor binding was essentially complete after 20–30 min incubation (Fig. 2) and that nonspecific binding increased significantly after 60 min. Therefore, an incubation time of 60 min was selected. The saturation of the system is demonstrated in Fig. 1, and Fig. 3 provides an example of the displacement of radioactive ligand from the binding sites. In this system, receptors are saturable and the binding can be displaced by either adrenergic agonists or antagonists. The assay exhibited identical characteristics for both β-1 and β-2 receptors.

Table I gives the relative binding of some reference compounds which are in agreement with the previous published data (1). The conditions of the assay proved to be highly stereospecific and selective for both β-1 and β-2

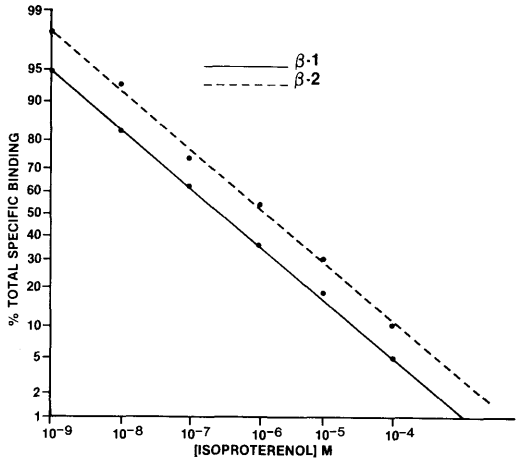

FIG. 3. β-1 and β-2 adrenergic receptor membranes: Displacement of [^{3}H]DHA by (I) isoproterenol. The results are the means of three separate experiments (SEM in each case < ±5%).

binding (Table I). None of the other compounds tested yielded binding significantly different from nonspecific binding (Table I).

Finally we applied the assay to the measurement of β-1 and β-2 binding affinity of some of our synthetic adrenergic agents and

TABLE I. STEREOSPECIFICITY AND SELECTIVITY OF THE ASSAY

Compound	β-1 binding	β-2 binding
L-Isoproterenol	100	100
D-Isoproterenol	7	8
DL-Isoproterenol	73	59
L-Epinephrine	7	25
D-Epinephrine	1	2
DL-Epinephrine	6	13
L-Dopa	—	—
Dopamine	—	—
Haloperidol	—	—
DL-Norepinephrine	8	—
5-Methoxytyramine	—	—
DL-Normetanephrine	—	—
DL-Metanephrine	—	—
5-Hydroxytryptophan	—	—
5-Hydroxytryptamine	—	—
Melatonin	—	—
Phentolamine	—	—
DL-Propranolol	1760	550

Note. A value of 100 is assigned arbitrarily to l-isoproterenol binding. Up to 100 μ*M* concentration of each compound was tested. (—), no binding.

TABLE II. COMPOUNDS WITH DESIRED ACTIVITIES

CL Number	β-1 K-50 (μ*M*)	β-2 K-50 (μ*M*)
1. 263,780	1.412 ± 0.08	1.044 ± 0.05
2. 252,770	9.130 ± 0.91	0.930 ± 0.03
3. 271,662	16.644 ± 1.52	1.864 ± 0.07
4. 266,206	61.940 ± 6.32	0.804 ± 0.06
5. 269,809	11.030 ± 0.98	1.128 ± 0.09
6. 266,611	22.056 ± 1.91	2.083 ± 0.19
7. 266,625	29.744 ± 3.25	2.095 ± 0.25
8. 263,427	21.123 ± 1.86	2.289 ± 0.19

Note. β Adrenergic activity is inversely related to K-50 (the concentrations of the compound required to displace 50% radioactive ligand from binding sites of β adrenergic receptors) value. Mean ± SEM, $n = 5$.

the results are given in Table II. Thus we have described a competitive receptor binding radioassay for β-1 and β-2 adrenergic analogs.

Discussion. Even though several iodinated and tritiated adrenergic agonists and antagonists are commercially available (14), the β adrenergic antagonist [^{3}H]DHA was used, since it can be obtained as the pure R stereoisomer. When racemic ligands are used, problems may arise since the individual stereoisomers may show differences in both their kinetic and their equilibrium binding properties (15, 16). Displacement curves of antagonists competing for antagonist ligands are steep, whereas the displacement curves of agonists are shallow (17), suggesting that agonists bind to both high and low affinity forms of the β receptor. These high and low affinity forms appear to be interconvertible and the agonist seems to induce the high affinity form upon binding to the β receptor, thus adding to the measure of affinity. The use of [^{3}H]DHA obviates the major problems of radioiodinated ligands, which are the generation of "damaged" but labeled molecules during the iodination procedures, a greater susceptibility to radiation-induced destruction of molecular structure (decay catastrophe), different behavior of the iodinated substance in analytical systems compared to the parent molecule, and a short radiochemical half-life of ^{125}I (60–70 days). On the other hand, [^{3}H]DHA is biologically indistinguishable from the native compound and has a relatively high specific activity (100 Ci/mmole). Purification of [^{3}H]DHA is

simple since it behaves in a manner identical to the unlabeled compound in analytical systems (18). It is very stable with a radiochemical half-life of greater than 12 years. Furthermore, DHA has been shown to possess high affinity and biological specificity for the β adrenergic receptor in several well-characterized membrane preparations (18).

The separation of bound radioactivity from the unbound part is accomplished by filtration and washing on Whatman GF-B glass filters. This method is rapid and minimizes the risk of ligand dissociation from the receptors. Centrifugation techniques used for the same purpose seem to be slower and give rise to higher nonspecific binding. The ligand also binds to components in the membrane other than receptors which gives rise to nonspecific binding. Nonradioactive dihydroalprenolol and isoproterenol do not interfere with the nonspecific binding. Nonspecific binding was estimated by incubating the membranes in the presence of an excess of unlabeled dihydroalprenolol or isoproterenol. Siliconizing the glass fiber filter reduced filter binding. Low nonspecific binding will increase assay accuracy.

In conclusion, the receptor binding radioassay described for β-1 and β-2 adrenergic agent is simple, sensitive, inexpensive, efficient, and requires minimal manual support. It is suitable for screening synthetic adrenergic compounds for their selective β-1 and β-2 activities. The use of the Brandel M-48R cell harvester for filtration allows a technician to process 50 samples in a single working day.

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