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Comparison of Two Putatively Selective Radioligands for Labeling Central Nervous System β-Adrenergic Receptors: Inadequacy of [^{3}H]Dihydroalprenolol

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SUMMARY

[^{3}H]Dihydroalprenolol ([^{3}H]DHA) has been used extensively in receptor binding studies to measure β-adrenergic receptors in the central nervous system. Usually, nonspecific binding has been defined by high concentrations of the β-adrenergic receptor agonist isoproterenol or antagonists such as alprenolol or propranolol. Scatchard plots of such "specific" [^{3}H]DHA saturation data in rat cerebral cortex membranes are linear. However, computer analysis demonstrated that the competition curves of these drugs for 2.0 nM [^{3}H]DHA binding are biphasic, with a continuous inhibition of [^{3}H]DHA binding in the concentration range usually used to determine nonspecific binding. These data indicate that another saturable high affinity site was being labeled by the radioligand and that the definition of nonspecific binding with any of these unlabeled drugs is not satisfactory. We used the nonlinear, least squares, curve-fitting program LIGAND to analyze total [^{3}H]DHA binding, allowing the program to mathematically define nonspecific binding as a function of ^{3}H-ligand concentration. Significantly lower B_{max} (−44%) and K_d (−58%) values for β-adrenergic receptors were found, indicating that under normal experimental procedures (defining [^{3}H]DHA nonspecific binding with these nonradioactive drugs) a second binding site was being labeled. We found that [^{3}H]DHA binding to this site could be inhibited by drugs such as RU24969, a 5-hydroxytryptamine$_{1A}$ (5HT$_{1A}$) and 5HT$_{1B}$ receptor subtype-selective agonist, and CGS12066B, a 5HT$_{1B}$ receptor subtype-selective agonist, which were able to compete for 15–20% of [^{3}H]DHA binding in the nanomolar concentration range, whereas drugs that are selective for other serotonin receptor subtypes inhibited [^{3}H]DHA binding only at much higher concentrations. Another β-adrenergic receptor antagonist radioligand, [^{3}H]CGP-12177, was found to be more selective for β-adrenergic receptors. Alprenolol competition curves for [^{3}H]CGP-12177 binding were monophasic and saturation curves, with nonspecific binding defined either by 10 μM alprenolol or by LIGAND, yielded B_{max} values close to those obtained with [^{3}H]DHA when its nonspecific binding was defined by LIGAND. [^{3}H]DHA cannot be considered a suitable radioligand to quantify central nervous system β-adrenergic receptors in the manner in which it has been typically used.

[^{3}H]DHA was one of the first radioligands used to identify peripheral β-adrenergic receptors (1, 2). This ligand was then used to identify β-adrenergic receptor sites in the mammalian CNS (3) and has been used in many studies since, especially those investigating the regulation of β-adrenergic receptors by pharmacological treatments with antidepressant drugs (4, 5). Although it is known that the proportions of β_1 and β_2 subtypes of adrenergic receptors vary between brain regions (6, 7), most studies do not differentiate between the subtypes and consider that [^{3}H]DHA labels both subtypes with equal affinity. In fact, it is known that [^{3}H]DHA has a slightly higher affinity for β_2- versus β_1-adrenergic receptors (8).

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Stone and U'Prichard (9) suggested that [^{3}H]DHA may also label other sites besides β-adrenergic receptors in the rat brainstem, although they did not characterize them or investigate other brain regions. The possibility that other sites are also labeled in the cortex is reinforced by the independent observations of Sulser and Kellar and their co-workers (10, 11), who have found that alterations in β-adrenergic receptor-linked adenylate cyclase activity do not necessarily parallel changes in β-adrenergic receptors measured with [^{3}H]DHA and that manipulations of serotonergic systems can alter the binding characteristics of this ligand. Recently, other β-adrenergic ligands, which were introduced and characterized after [^{3}H]DHA, have been shown to label serotonin receptors (12, 13).

The specificity of the receptor binding of a radioligand is

ABBREVIATIONS: [^{3}H]DHA, [^{3}H]dihydroalprenolol hydrochloride; CGP-12177, (−)-4-(3-*tert*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one; 8-OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)tetraline HBr; RU24969, 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl) indole; CGS-12066B, 7-trifluoromethyl-4(4 methyl-1-piperazinyl)-pyrrolo[1,2a]quinoxaline dimaleate salt; ICI 89,406, 1-(2-cyanophenoxy)-3-β-(3-phenylureido)ethyl-amino-2-propanolol; CNS, central nervous system; 5HT, 5-hydroxytryptamine; ICI 118,551,erytro-dl-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol.

dependent upon two characteristics, 1) the differential affinities of the radioligand for its various binding sites and 2) the selectivity and affinities of competing nonradioactive drugs that are used to saturate specific receptor sites and, hence, define "nonspecific" or nonreceptor binding of the radioligand. In the case of [^{3}H]DHA, micromolar or higher concentrations of the β-adrenergic receptor antagonists alprenolol or propranolol have generally been used to define its nonspecific binding. However, these drugs have been shown, in studies with other radioligands, to have some affinity for serotonergic receptor sites (14, 15).

Recently, it has become possible to analyze the receptor binding of a radioligand directly, without the use of a high concentration of a nonradioactive drug to define nonspecific binding. This method of analysis makes use of the iterative, nonlinear regression, curve-fitting program LIGAND, which fits saturation binding data, according to the law of mass action, for the binding of the radioligand to one or more saturable (putative receptor) sites plus nonspecific binding, which is fitted as a linear function of radioligand concentration (16). This method provides major advantages over the typically used Scatchard analysis (17, 18). In competition studies, where increasing concentrations of a nonradioactive drug compete for the binding sites occupied by a fixed concentration of radioligand, the program LIGAND can also be used to analyze the binding of both the radioactive and nonradioactive ligands to single or multiple sites, with nonspecific binding either defined experimentally (by a high concentration of any nonradioactive drug) or fitted by the program to the competition curve under analysis.

In this paper, we have reevaluated the binding of [^{3}H]DHA to CNS receptor sites, defining "nonspecific" binding by LIGAND or by use of drugs that have been commonly used in other investigations, and we compared the results with those of a novel β-adrenergic receptor radioligand, [^{3}H]CGP-12177 (19). Our conclusion is that [^{3}H]DHA is not suitable for measuring β-adrenergic receptors as it has been used typically and the other site(s) that it labels cannot be easily characterized.

Materials and Methods

Animals and tissue preparation. Adult male Sprague Dawley rats (Charles River, Wilmington, MA), weighing 200–300 g, were used throughout the experiments; they were housed in group cages under standard conditions (12-hr light–dark cycle) with free access to food and water. The animals were killed by decapitation (between 10 a.m. and 1 p.m.) and the brains were immediately removed into ice-cold saline. Brain regions were rapidly dissected, placed into plastic vials, frozen on dry ice, and stored at −70° until the day of the experiment. Rat cerebral cortices were homogenized in 50 volumes of cold 50 mM Tris·HCl (pH 7.7 at 25°) and centrifuged three times at 35,000 × g for 20 min.

β-Adrenergic receptor binding. Two different ligands were used to measure β-adrenergic receptor binding to rat cerebral cortex membranes, [^{3}H]DHA (specific activity, 52.3–95 Ci/mmol; New England Nuclear, Boston, MA) and [^{3}H]CGP-12177 (specific activity, 53 Ci/mmol; Amersham Corporation, Arlington Heights, IL). Binding was performed in borosilicate disposable tubes. The final assay volume of 2 ml consisted of 100 μl of radiolabeled ligand, 100 μl of 10 μM alprenolol, propranolol, or isoproterenol to measure the "nonspecific" binding, competing drug as required, or incubation buffer (50 mM Tris·HCl, pH 7.7 at 25°), 800 μl of incubation buffer, and 1 ml of membrane suspension (6 mg of wet weight tissue/tube) added at the start of the incubation.

The test tubes were incubated for 30 min at 37°, then filtered under vacuum through Whatman GF/C filters, and washed three times with 5 ml of ice-cold Tris buffer, using a modified Brandel cell harvester. Filters were placed in plastic mini-scintillation vials and 5 ml of Ecolume (ICN Biomedicals, Inc.) were added. Radioactivity trapped on the filters was counted using a Beckman LS 5000 TD scintillation counter at an efficiency of 43%.

In saturation experiments, [^{3}H]DHA was usually incubated in a concentration range of 0.2 to 6.0 nM, whereas 2.0 nM [^{3}H]DHA was used in competition experiments unless stated otherwise. In a typical experiment, at K_d concentration the total and nonspecific (defined by 10 μM alprenolol) binding for [^{3}H]DHA were 2183 and 471 cpm.

Saturation curves of [^{3}H]CGP-12177 were performed in a concentration range of 0.025 to 2.0 nM, whereas in competition studies 0.2 nM [^{3}H]CGP-12177 was used. In a typical experiment, at K_d concentration the total and nonspecific (defined by 10 μM alprenolol) binding for [^{3}H]CGP-12177 were 679 and 51 cpm.

Data analysis. The weighted, nonlinear least squares, curve-fitting program LIGAND was used for the analysis of saturation and competition experiments (16). Saturation experiments were analyzed either by fitting the specific binding (defined as the difference between total binding and nonspecific binding, which was delineated by the competing nonradioactive drug) or by fitting the total binding with the nonspecific binding allowed to float and being fitted by the program as a linear function of ^{3}H-ligand concentration. All saturation and competition studies were initially analyzed with a one-site model (i.e., one saturable binding site); the data were then analyzed according to a two-site model (i.e., two distinct saturable binding sites of different affinities) and the results of this curve fitting were statistically compared with a one-site model by an F test. The two-site model was accepted if the fit was significantly better ($p < 0.05$) with respect to the one-site analysis. Nonspecific binding in the competition experiments either was fixed to the value defined by 10 μM alprenolol or was determined by LIGAND from curve fitting of the competition of the nonradioactive drug under examination. LIGAND also allows for the simultaneous analysis of multiple competition curves for the same ligands, providing more information for the accurate calculation of binding parameters.

When comparisons between assays with different definitions of nonspecific binding were made, an ANOVA test was used and a direct comparison between any two groups was made by Dunnett t test. When comparisons were made between assays run in parallel on the same tissue samples, comparisons were also made by paired Student's t test.

Drugs. The following drugs were donated: CGP-12177 (Ciba Geigy), RU24969 (Dr. S. Peroutka, Stanford University or Roussel), metergoline (Dr. L. Meyersen, Lederle), mesulergine (Dr. S. Peroutka, Stanford University or Sandoz), ketanserin tartrate and spiperone (Janssen Pharmaceutica), quipazine maleate (Dr. S. Peroutka, Stanford University), and ICI 89,406 (Imperial Chemical Industries).

The following compounds were purchased: [^{3}H]DHA (NEN Dupont), (−)-[^{3}H]CGP-12177 (Amersham), l-alprenolol d-tartrate, l-(−)-isoproterenol d-bitartrate, GTP Tris salt and DL-propranolol hydrochloride (Sigma), and 8-OH-DPAT and CGS-12066B (Research Biochemicals Incorporated).

Results

Characterization of [^{3}H]DHA binding. Alprenolol and propranolol have been used in many studies to define nonspecific binding of [^{3}H]DHA to β-adrenergic receptors. A concentration of 1–10 μM has been used typically. To ensure that only a single homogeneous population of receptor binding sites is being identified, the competition curve of these drugs should have a pseudo-Hill slope of 1.0 and concentrations of the competing drug higher than the concentration used to define nonspecific binding should not inhibit the binding of the radioligand further. Fig. 1 shows a typical competition curve of alprenolol for [^{3}H]DHA binding. As can bee seen, competition

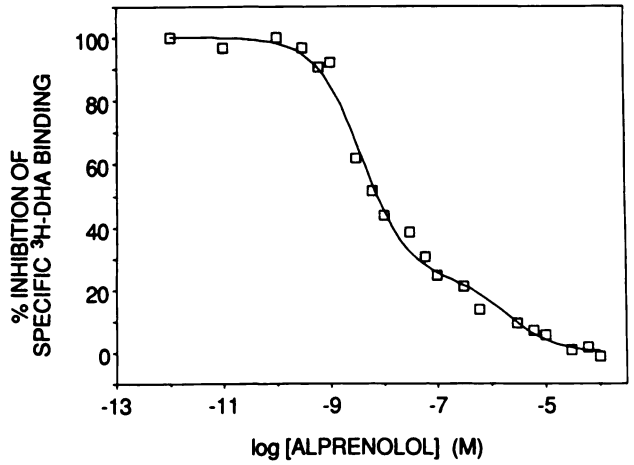

Fig. 1. Typical competition curve of alprenolol for [^{3}H]DHA binding in rat cerebral cortex membranes. The concentration of [^{3}H]DHA was 2.0 nM. Nonspecific binding was defined by the curve-fitting program LIGAND and it was determined to be 18% of total [^{3}H]DHA binding. The alprenolol competition curve fit best to a two-site model ($p < 0.001$), with K_H = 1.65 ± 0.18 nM and K_L = 7.37 ± 2.35 μM (mean ± SE of 12 separate competition experiments) when the affinity of [^{3}H]DHA for the high affinity β-adrenergic receptor site was fixed at 0.6 nM and its affinity for its second binding site was defined by LIGAND. The affinity of [^{3}H]DHA determined for the second site was 6.64 ± 0.71 nM (see text for explanation). The densities obtained for the two sites were R_H = 6.26 ± 0.25 fmol/mg of tissue and R_L = 6.47 ± 0.53 fmol/mg of tissue.

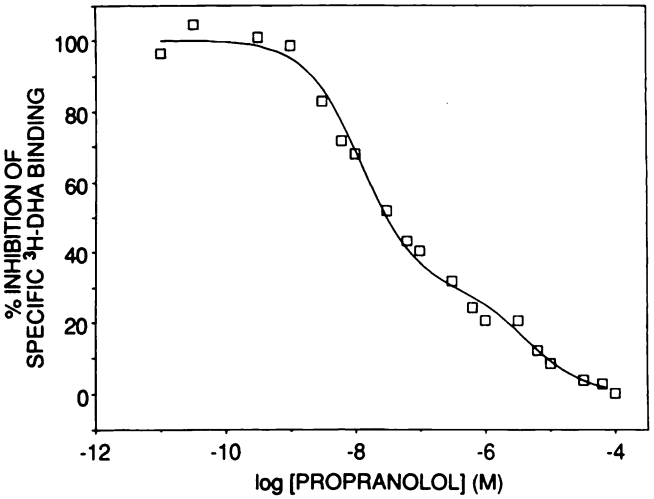

Fig. 2. Typical competition curve of propranolol for [^{3}H]DHA binding in rat cerebral cortex membranes. The concentration of [^{3}H]DHA was 2.0 nM. Nonspecific binding was defined by LIGAND and it was determined to be 22% of total [^{3}H]DHA binding. The curve was best fit to a two-site model ($p < 0.001$), with K_H = 2.94 ± 0.51 nM and K_L = 2.31 ± 0.91 μM. These values were obtained setting the affinity of [^{3}H]DHA for the two sites at 0.6 nM. When the affinity of [^{3}H]DHA for the second site was set at 6.64 nM, as obtained from [^{3}H]DHA/alprenolol competition curves, the determined affinities of propranolol for the two sites were K_H = 2.91 ± 0.53 nM and K_L = 7.79 ± 3.18 μM, whereas the capacities of the two sites were R_H = 5.36 ± 0.32 and R_L = 6.95 ± 0.28 fmol/mg of tissue.

of alprenolol for [^{3}H]DHA binding is steep between 0.1 and 100 nM, at which point the curve tends to level off but continues to decrease gradually to the highest concentration studied, 100 μM. Fig. 2 shows a typical competition curve of propranolol for [^{3}H]DHA binding; also with this drug a continuous inhibition of the binding occurs in the concentration range currently used to define the nonspecific binding of [^{3}H]DHA. The shape of these curves suggests that more than one site is being labeled. Furthermore, it suggests that no concentration of alprenolol or propranolol can satisfactorily be used to define [^{3}H]DHA specific binding to β-adrenergic receptors.

In order to analyze these curves by LIGAND, it is first necessary to determine the affinity of [^{3}H]DHA for the binding sites that it is identifying. This is done by conducting saturation experiments investigating the binding of increasing concentrations of [^{3}H]DHA. If nonspecific binding was defined by 10 μM alprenolol, Scatchard analysis of such "specific" [^{3}H]DHA binding indicated binding to a homogeneous population of sites with a K_d of 1.48 ± 0.10 nM and a B_{max} of 9.86 ± 0.33 fmol/mg of tissue (16 experiments) (Fig. 3A). Analysis of the same data (nonspecific binding defined by 10 μM alprenolol) with LIGAND yielded a best fit to a single-site model, with a K_d of 1.42 ± 0.10 nM and a B_{max} of 9.61 ± 0.33 fmol/mg of tissue (16 experiments) (see Table 1). LIGAND and Scatchard analysis of [^{3}H]DHA saturation curves, with nonspecific binding defined by 10 μM propranolol, yielded similar although significantly higher values, with a K_d of 2.31 ± 0.26 nM and a B_{max} of 11.67

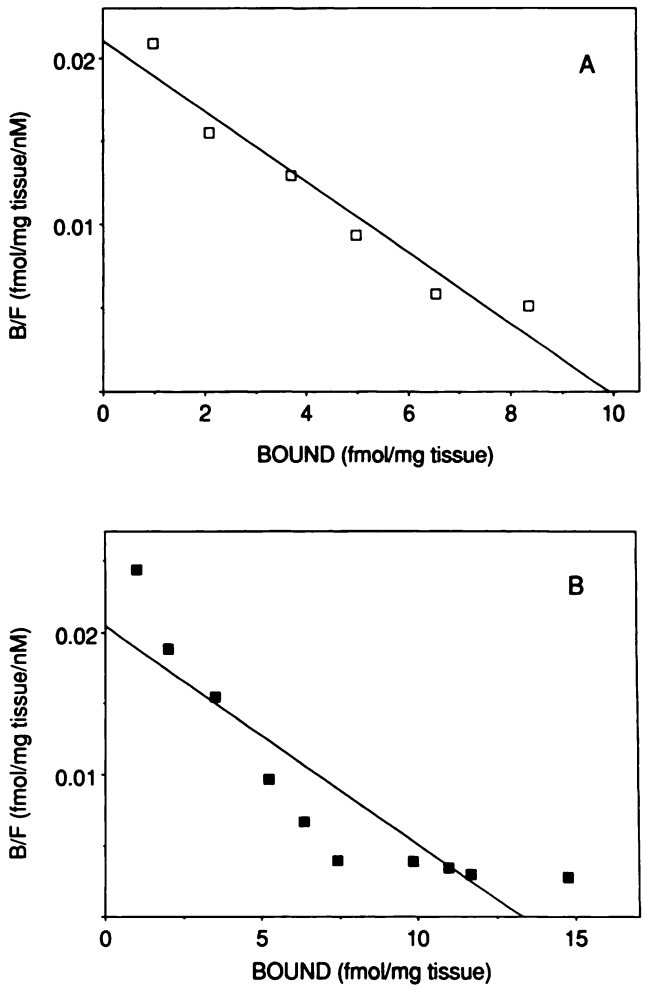

Fig. 3. Scatchard plots of typical [^{3}H]DHA saturation experiments in rat cerebral cortex membranes with nonspecific binding defined by 10 μM alprenolol. The *lines* were calculated by linear least-squares analysis. A, [^{3}H]DHA was used in a concentration range of 0.2 to 5.0 nM. The Scatchard plot for a single site (correlation coefficient, r = 0.97) yielded a K_d of 1.41 nM and a B_{max} of 9.88 fmol/mg of tissue. B, The concentration range of [^{3}H]DHA was between 0.2 and 16 nM. The Scatchard plot for a single site (correlation coefficient, r = 0.89) yielded a K_d of 1.94 nM and a B_{max} of 13.08 fmol/mg of tissue.

TABLE 1

Nonlinear regression analysis (LIGAND) of saturation data for [^{3}H]DHA and [^{3}H]CGP-12177 binding to β-adrenergic receptors in rat cerebral cortex membranes

Saturation experiments were performed as described in Materials and Methods and were analyzed by the computer program LIGAND. All data were best fit to a single saturable site; the values in the table are the means ± standard error of *n* separate determinations.

^{3}H-Ligand	*n*	Nonspecific binding defined by	B_{max}	K_d
			fmol/mg of tissue	*nM*
[^{3}H]DHA	16	10 μM Alprenolol	9.61 ± 0.33	1.42 ± 0.10
[^{3}H]DHA	4	10 μM Isoproterenol	6.04 ± 0.22[a]	0.95 ± 0.21
[^{3}H]DHA	7	10 μM Propranolol	11.67 ± 0.66[b]	2.31 ± 0.26[b]
[^{3}H]DHA	16	LIGAND program	5.42 ± 0.19[a,c]	0.60 ± 0.05[d]
[^{3}H]CGP-12177	12	10 μM Alprenolol	4.99 ± 0.12[e]	0.13 ± 0.01
[^{3}H]CGP-12177	12	LIGAND program	4.46 ± 0.18[e,f]	0.10 ± 0.01

[a] $P < 0.001$ versus [^{3}H]DHA binding in which the nonspecific binding was determined by 10 μM alprenolol or 10 μM propranolol (Dunnett *t* test).
[b] $p < 0.01$ versus [^{3}H]DHA binding in which the nonspecific binding was determined by 10 μM alprenolol (Dunnett *t* test).
[c] $p < 0.001$ versus [^{3}H]DHA binding in which the nonspecific binding was determined by 10 μM alprenolol (paired *t* test on 16 pairs run in parallel).
[d] $p < 0.05$ versus [^{3}H]DHA binding in which the nonspecific binding was determined by 10 μM alprenolol (paired *t* test on 16 pairs run in parallel).
[e] $p < 0.01$ versus [^{3}H]DHA binding in which nonspecific binding was determined either by 10 μM isoproterenol or by LIGAND program (Dunnett *t* test).
[f] $p < 0.05$ versus [^{3}H]CGP-12177 binding in which the nonspecific binding was determined by 10 μM alprenolol (paired *t* test on 12 pairs run in parallel).

± 0.66 fmol/mg of tissue. However, if nonspecific binding was not defined by a competing nonradioactive drug and the program LIGAND was allowed to fit the total binding of [^{3}H]DHA to one or more saturable sites with nonspecific binding as a linear function of [^{3}H]DHA concentration, only a single saturable binding site could be identified, with a K_d of 0.60 ± 0.05 nM ($p < 0.05$ versus [^{3}H]DHA binding with nonspecific binding defined by 10 μM alprenolol) and a significantly lower B_{max} of 5.42 ± 0.19 fmol/mg of tissue ($p < 0.001$) (see Table 1).

We used the K_d determined from the saturation data by LIGAND (with nonspecific binding determined by the program) to reanalyze the competition curves of alprenolol and propranolol for [^{3}H]DHA binding that are shown in Fig. 1 and 2. With both drugs, a two-site fit was significantly better than a one-site fit ($p < 0.001$), with values for the affinities of alprenolol being $K_H = 1.65 \pm 0.18$ nM and $K_L = 2.52 \pm 0.81$ μM, with 78 ± 2% representing the high affinity sites; the parameters for propranolol were $K_H = 2.94 \pm 0.51$ nM, $K_L = 2.31 \pm 0.91$ μM, and 72 ± 1% was represented by the high affinity site. For this analysis, the affinity of [^{3}H]DHA for binding to the second site was fixed to be the same as the affinity determined by LIGAND for the first site (presumptive β-adrenergic receptors), because Scatchard or LIGAND analysis had resolved only a single homogeneous population of [^{3}H]DHA binding sites.

It was obvious, however, from the analysis of the saturation data comparing the results using nonspecific binding either defined by 10 μM alprenolol or being calculated by LIGAND that the affinity of [^{3}H]DHA for the second site must be lower than its affinity for the β-adrenergic receptor sites because 1) the K_d for the single highest affinity site (presumptive β-adrenergic receptors) determined by LIGAND analysis of total binding was significantly lower than the K_d determined by LIGAND or Scatchard analysis when nonspecific binding was determined by 10 μM alprenolol, which would have included binding to the second site in the analysis; and 2) when increasing concentrations of [^{3}H]DHA were included in the experiment in an attempt to determine the characteristics of the second site, the Scatchard plots became curvilinear and showed continual increases in K_d and B_{max} when the data were analyzed as binding to a single site (Fig. 3B). However, it was impossible for LIGAND to resolve specific binding, either defined by 10 μM alprenolol or from total binding, into two saturable sites (plus nonspecific binding). In all cases, only a single saturable site could be defined. For example, if concentrations of up to 20 nM [^{3}H]DHA were included, LIGAND still analyzed the total binding data as a single saturable binding site with the same B_{max} and K_d as were determined with saturation curves when the highest concentration of [^{3}H]DHA was 6 nM. Thus, the second site appeared not to saturate under the conditions of the assay and LIGAND considered that the binding to it was linear with increasing radioligand concentration and included it in the determination of nonspecific binding. Whether or not the second site, in reality, is not saturable is uncertain because, at these high concentrations of [^{3}H]DHA, nonspecific binding was so great that if the B_{max} of the second site is not large (in the same order as that of other neurotransmitter receptors, about 10 fmol/mg of wet weight tissue) the noise in the data would obviate its accurate determination by LIGAND.

Given that it appeared that the affinity of [^{3}H]DHA for the second site was lower than that for β-adrenergic receptors, we reanalyzed the [^{3}H]DHA/alprenolol competition curves, letting LIGAND determine the affinities of alprenolol for both the β-adrenergic receptor and the second site, while fixing only the affinity of [^{3}H]DHA for the β-adrenergic receptor population as determined by LIGAND analysis of the saturation data. The affinity of [^{3}H]DHA for the second site and its B_{max} values for the two sites were allowed to float and be calculated by LIGAND. Under these conditions, LIGAND again determined that a two-site fit for the competition curve for alprenolol was significantly better than a single-site fit. However, this fit was not significantly better from the two-site fit where the affinities of [^{3}H]DHA for the β-adrenergic receptor and the second site were shared and set to the value determined for the β-adrenergic receptor determined from the saturation studies. Although this fit was not significantly better, the K_d that was determined may give some approximation of the affinity of [^{3}H]DHA for the second site. The average value that was determined (12 experiments) for the affinity of [^{3}H]DHA for the second site was 6.64 ± 0.71 nM and the B_{max} for this site was 6.47 ± 0.53 fmol/mg of tissue.

Isoproterenol, a β-adrenergic receptor agonist, has also been used in some studies to define nonspecific binding. However, a problem with using agonists to define nonspecific binding is

that in certain circumstances, especially if the receptor is associated with a guanine nucleotide-binding protein, agonists can demonstrate both high and low affinity binding components. Thus, it can be unclear whether only the highest affinity component defined by the agonist competition represents the specific receptor binding or whether it is the two highest affinity components that define the total population of receptors. Indeed, as will be mentioned in the discussion, this had lead to a good deal of confusion in the analysis of some experiments. When we defined nonspecific binding by 10 μM isoproterenol and conducted a saturation of [^{3}H]DHA binding, we obtained a B_{max} that was close to the value determined by LIGAND analysis of total binding with nonspecific binding calculated as a linear function of ^{3}H-ligand concentration (see Table 1), suggesting that β-adrenergic receptors, under these conditions, demonstrate only a single affinity binding state. Competition curves of isoproterenol for [^{3}H]DHA binding (Fig. 3) were best fit by a two-site model, with the highest affinity component having a K_i of 50.0 ± 3.7 nM and a B_{max} of 4.56 ± 0.32 fmol/mg of tissue, similar to the B_{max} of β-adrenergic receptors as defined by saturation experiments. The second site had a much lower affinity (greater than 1 μM) and its B_{max} approximated the B_{max} for the second site as defined by alprenolol competition curves. In order to conduct this analysis, LIGAND was used to analyze the competition curve for two sites and the affinity of [^{3}H]DHA for the second site was fixed to the same value as its highest affinity site (β-adrenergic receptors) or the affinity of the second site was set to 6.64 nM, as had been determined from the alprenolol competition analysis by LIGAND (Fig. 4).

Isoproterenol binding at both of the sites labeled by [^{3}H]DHA was sensitive to GTP. The curves were shifted to the right and best fit to a single-site model in the presence of 300 μM GTP. The affinity of isoproterenol for the high affinity binding site was lowered by a factor of 3, whereas the second component, for which in the absence of GTP isoproterenol had an affinity of 9.18 ± 1.79 μM, was not apparent in the presence of GTP. Isoproterenol at 10 μM competed for only 57% of total [^{3}H]DHA binding in the presence of 300 μM GTP versus 70% of total [^{3}H]DHA binding in the absence of GTP. The total binding of [^{3}H]DHA was not affected by 300 μM GTP. Importantly, the B_{max} for the highest affinity site for isoproterenol (4.56 ± 0.32 fmol/mg of tissue) was not significantly altered by 300 μM GTP (5.26 ± 0.46 fmol/mg of tissue).

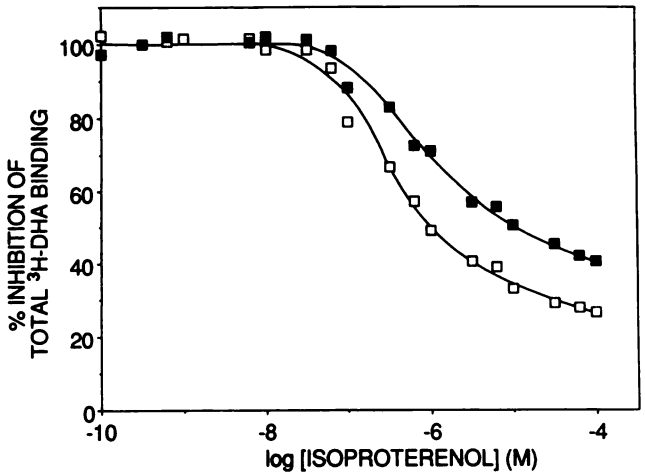

Fig. 4. Typical competition curve of isoproterenol on total [^{3}H]DHA binding in the presence (■) or absence (□) of 300 μM GTP. The curves (nine experiments) were analyzed by LIGAND and nonspecific binding was defined by the program. The concentration of [^{3}H]DHA was 2.0 nM. Specific binding was 70% of total in the absence of GTP and 57% in the presence of GTP. The competition curves of isoproterenol on [^{3}H]DHA binding in the absence of GTP were best fit to a two-site model ($p < 0.01$) with K_H = 50.0 ± 3.7 nM and K_L = 7.18 ± 1.79 μM. The capacities of the two sites (fmol/mg of tissue) were R_H = 4.56 ± 0.32 and R_L = 6.38 ± 0.72. These parameters were obtained by fixing the affinity of [^{3}H]DHA for the high affinity site at 0.6 nM while its affinity for the second site was set at 6.64 nM (the value obtained from alprenolol competition curves). The competition curves in the presence of GTP were best fit to a one-site model, with K_i = 174.5 ± 10.0 nM ($p < 0.01$ versus K_H of isoproterenol in the absence of GTP) and a density of 5.26 ± 0.46 fmol/mg of tissue.

Characterization of [^{3}H]CGP-12177 binding. We then characterized the binding of another β-adrenergic antagonist radioligand, [^{3}H]CGP-12177. When [^{3}H]CGP-12177 saturation curves were conducted using 10 μM alprenolol to define nonspecific binding, LIGAND analysis yielded a B_{max} of 4.99 ± 0.12 fmol/mg of tissue. This value was not significantly different from the B_{max} for [^{3}H]DHA binding calculated by LIGAND with nonspecific binding as defined by the computer program (Table 1). If LIGAND was used to define nonspecific binding for [^{3}H]CGP-12177, the B_{max} was 11% lower than if 10 μM alprenolol was used to define nonspecific binding and it was calculated as 4.46 ± 0.18 fmol/mg of tissue ($p < 0.05$, compared with the alprenolol-defined B_{max}; Table 1). This value was close to, although significantly lower ($p < 0.01$, 12 experiments) than, the B_{max} calculated for [^{3}H]DHA binding using LIGAND to define nonspecific binding.

The competition of alprenolol for [^{3}H]CGP-12177 (Fig. 5) indicates that [^{3}H]CGP-12177 is a more specific β-adrenergic receptor radioligand than is [^{3}H]DHA. The LIGAND analysis of this competition data was best fit to a single site with high affinity for alprenolol (K_i = 1.80 ± 0.16 nM) and the competition curve leveled off between 0.1 and 100 μM alprenolol. Thus, alprenolol would appear to be a satisfactory drug to define the nonspecific binding of [^{3}H]CGP-12177 to β-adrenergic receptors.

In contrast to the competition of isoproterenol for [^{3}H]DHA binding, where two sites were apparent, isoproterenol competition for [^{3}H]CGP-12177 binding was best fit to a one-site

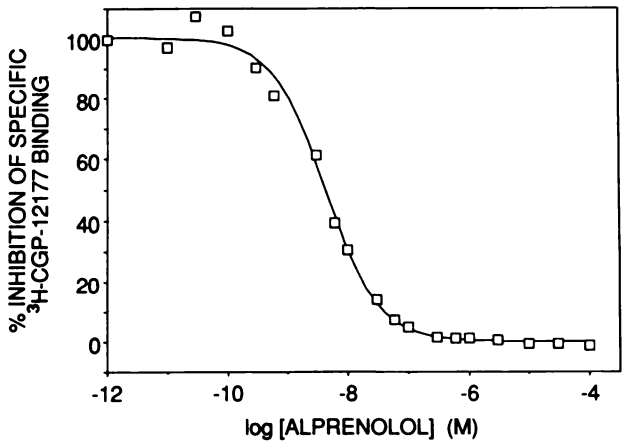

Fig. 5. Typical competition curve of alprenolol for [^{3}H]CGP-12177 binding in rat cerebral cortex membranes. Alprenolol competition curves for 0.2 nM [^{3}H]CGP-12177 were monophasic, with a K_i = 1.80 ± 0.16 nM and a B_{max} = 5.16 ± 0.14 fmol/mg of tissue (mean ± SE of five separate competition experiments). Nonspecific binding was defined by LIGAND and amounted to 8% of total binding.

model, with a K_i of 132 ± 8 nM and a B_{max} of 5.17 ± 0.09 fmol/mg of tissue, which was not significantly different from the B_{max} of [^{3}H]CGP-12177 binding determined from the saturation experiments with nonspecific binding defined by alprenolol (Fig. 6). It is interesting to note that the affinity of isoproterenol for this single high affinity site was significantly lower ($p < 0.01$) than the affinity of isoproterenol for its high affinity site identified in [^{3}H]DHA binding experiments (132 versus 50 nM). GTP at 300 μM shifted the isoproterenol curve to the right and its K_i was increased by a factor of 2. Total [^{3}H]CGP-12177 binding was not altered by GTP, nor was the percentage of binding that was displaced by 100 μM isoproterenol (90.3 ± 0.7%).

Fig. 7 shows a typical competition curve of nonradioactive CGP-12177 for [^{3}H]DHA binding sites. LIGAND analysis of these competition curves was best fit by a three-site model ($p < 0.01$). The highest affinity component had a K_i of 0.13 ± 0.03 nM (not significantly different from the K_d of [^{3}H]CGP-12177 binding to β-adrenergic receptors in saturations experiments). The B_{max} of this site (4.56 ± 0.34 fmol/mg of tissue) was also identical to the B_{max} obtained in saturation experiments with [^{3}H]CGP-12177. The second component of the CGP-12177 competition for [^{3}H]DHA binding labeled a site with a K_i of 9.41 ± 1.95 nM and a B_{max} of 1.64 ± 0.28 fmol/mg of tissue. A third component of binding had a K_i of 47.7 ± 26.3 μM, when the affinity of [^{3}H]DHA for that site was set to the approximate affinity (as determined above) for the unknown site (6.64 nM). In this case, the B_{max} calculated for the third site was very variable, ranging from 1.95 to 5.86 fmol/mg of tissue (average, 3.82 ± 0.65 fmol/mg of tissue), because the highest concentration of CGP-12177 used (100 μM) was only slightly higher than the determined K_d for this site.

Characterization of the lower affinity [^{3}H]DHA binding site. Because studies by other authors had indicated that there was a potential serotonergic modulation of the lowest affinity site for [^{3}H]DHA binding defined by isoproterenol (20),

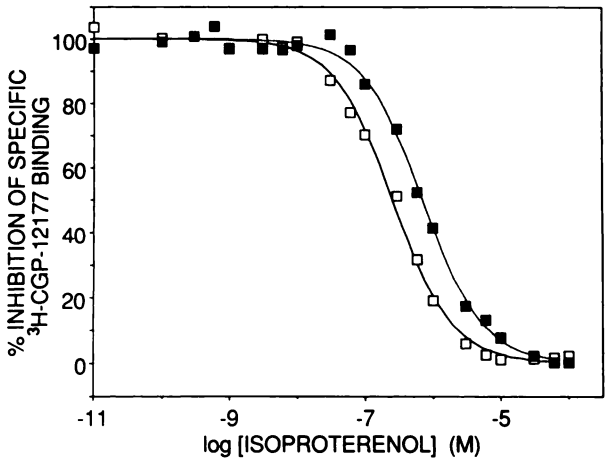

Fig. 6. Competition curve of isoproterenol on [^{3}H]CGP-12177 binding in rat cerebral cortex membranes: effect of GTP. The curves in the presence (■) or in the absence (□) of 300 μM GTP were analyzed by LIGAND and were best fit to a single-site model. In the absence of GTP, the K_i of isoproterenol for [^{3}H]CGP-12177 binding was 132 ± 8 nM and the measured density was 5.17 ± 0.09 fmol/mg of tissue, whereas in the presence of GTP the K_i was 263 ± 28 nM ($p < 0.01$ versus isoproterenol in the absence of GTP) and the density was 5.10 ± 0.17 fmol/mg of tissue. Specific binding was 90% of total binding in the presence or absence of GTP.

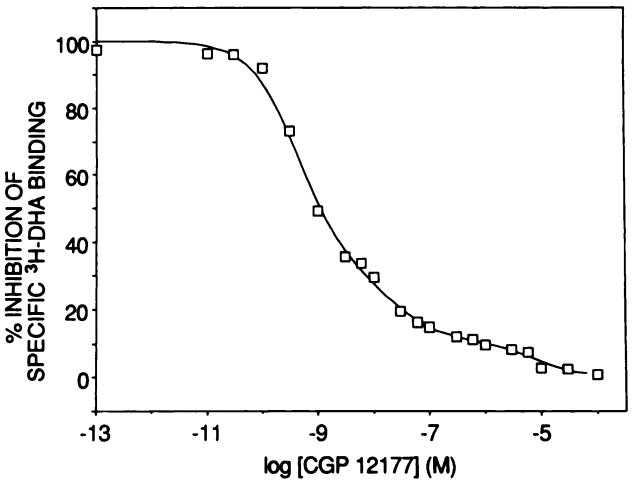

Fig. 7. Typical competition curve of [^{3}H]CGP-12177 on [^{3}H]DHA binding in rat cerebral cortex membranes. The concentration of [^{3}H]DHA was 2.0 nM. The curves, analyzed by LIGAND, fit best to a three-site model ($p < 0.01$), with nonspecific binding defined by the program. When the affinities of [^{3}H]DHA for the two highest affinity sites were set at 0.6 nM and its affinity for the third site was fixed at 6.64 nM, the determined K_i values for [^{3}H]CGP-12177 were: K_{i_1} = 0.13 ± 0.03 nM, K_{i_2} = 9.41 ± 1.95 nM, and K_{i_3} = 47.7 ± 26.3 μM. Their respective densities were R_1 = 4.56 ± 0.34, R_2 = 1.64 ± 0.28, and R_3 = 3.82 ± 0.65 fmol/mg of tissue.

we investigated a number of different serotonergic ligands of differing serotonin receptor subtype specificity for their ability to compete for [^{3}H]DHA binding. The selective $5HT_{1A}$ receptor ligand agonist 8-OH-DPAT, the semiselective $5HT_{1A}$ and $5HT_2$ antagonist spiperone, the selective $5HT_2$ receptor antagonist ketanserin, and the $5HT_{1C}$ receptor antagonist mesulergine only competed for [^{3}H]DHA binding at concentrations greater than 1 μM. The $5HT_{1B}$ receptor-selective agonist CGS12066B (21) and the semiselective $5HT_{1A}$/$5HT_{1B}$ receptor agonist RU24969 both competed for a portion of [^{3}H]DHA binding in the low nanomolar range. About 20% of 2.0 nM [^{3}H]DHA binding was displaced by 500 nM concentrations of these drugs (see Fig. 8). LIGAND analysis of these curves was best fit by a two-site model, with a high affinity component amounting to around 15–20% of "specific" [^{3}H]DHA binding as defined by 10 μM alprenolol. In order to better determine the characteristics of this high affinity site for the serotonergic ligands in the LIGAND analysis, we set the affinity of [^{3}H]DHA for this site as 6.64 nM (as determined from the alprenolol competition curves) and the affinity of [^{3}H]DHA for the β-adrenergic receptor as 0.6 nM. These data are shown in Table 2, along with the competition analyses of other selective serotonergic agonist/antagonists. The serotonergic drugs were also investigated for their ability to inhibit [^{3}H]CGP-12177 binding. None of the drugs that were active in a nanomolar range on [^{3}H]DHA binding inhibited [^{3}H]CGP-12177 binding at concentrations <100 nM.

As discussed above, CGP-12177 competed for three sites in [^{3}H]DHA binding experiments. In an attempt to see whether its lowest affinity site was the serotonergic site and whether it could be characterized in saturation studies, we masked the binding of [^{3}H]DHA to the two highest affinity sites competed for by CGP-12177, conducting a [^{3}H]DHA saturation experiment in the presence of 100 nM CGP-12177, with nonspecific binding defined by 1 μM CGS12066B. Scatchard and LIGAND analyses both yielded a similar K_d of 34 nM and a B_{max} of 16

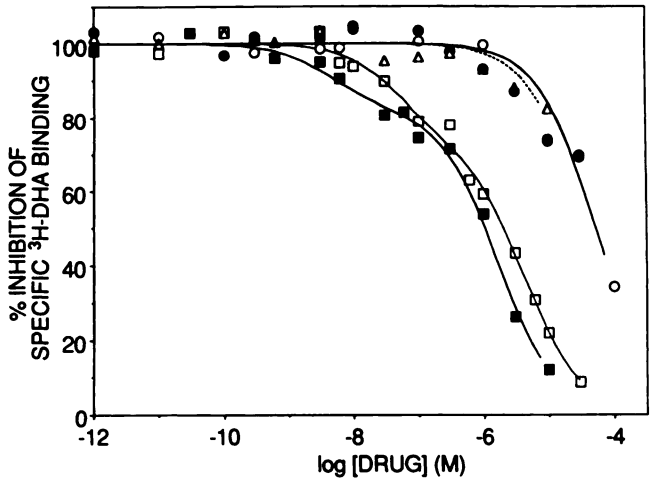

Fig. 8. Competition of serotonergic ligands for specific [^{3}H]DHA binding in rat cerebral cortex membranes. The concentration of [^{3}H]DHA was 2.0 nM. The data were analyzed by LIGAND, with nonspecific binding defined by 10 μM alprenolol. The competition curves of 8-OH-DPAT (△), mesulergine (○), and ketanserin (●) fit best to a single site, with their respective K_i values being 20.8, 7.2, and 17.1 μM. RU24969 (■) and CGS-12066B (□) competition curves were best fit to two sites ($p < 0.05$). The K_i values of RU24969 for the two sites were K_H = 29.4 ± 9.0 nM and K_L = 756 ± 92 nM, whereas the K_i values of CGS-12066B were K_H = 38.4 ± 14.2 nM and K_L = 576 ± 18 nM.

fmol/mg of tissue for [^{3}H]DHA binding to this site. However, not much reliance can be placed on these values, because specific binding was only 23% of total binding at the highest concentration (20 nM) of [^{3}H]DHA examined.

Resolution of [^{3}H]DHA and [^{3}H]CGP-12177 binding to β_1- and β_2-adrenergic receptors. It has previously been demonstrated that [^{3}H]DHA and [^{3}H]CGP-12177 bind to both β_1- and β_2-adrenergic receptors. The regional distributions of β_1-adrenergic and β_2-adrenergic receptors are not homogeneous, there being a greater proportion of β_1-adrenergic receptors in the cortex and β_2-adrenergic receptors in the cerebellum. Thus, the saturation curves conducted in the cortex will have binding parameters for the radioligands closer to their affinities for β_1-adrenergic receptors, whereas saturations conducted in the cerebellum will provide parameters approximating the affinities of the radioligands for β_2-adrenergic receptors. Some nonlabeled ligands are more selective for β_1- or β_2-adrenergic receptors than the classical β-adrenergic receptor antagonists; thus ICI 89,406 has a greater than 100-fold higher affinity for β_1- than β_2-adrenergic receptors (22), whereas ICI 118,551 has a more than 50-fold higher affinity for β_2- than β_1-adrenergic receptors (23). ICI 89,406 competed for [^{3}H]DHA binding in the cortex according to a three-site model; the highest affinity site had a K_i of 0.85 ± 0.18 nM and a receptor density of 3.46 ± 0.34 fmol/mg of tissue and the second site had a K_i of 111.7 ± 33.5 nM and a B_{max} of 1.71 ± 0.32 fmol/mg of tissue. When the K_d of [^{3}H]DHA for the third site was set at 6.64 nM, the B_{max} calculated for ICI 89,406 at this site was 6.70 ± 0.03 fmol/mg of tissue, similar to that obtained from alprenolol competition curves, and the affinity of ICI 89,406 for the third site was 23.4 ± 5.7 μM. On [^{3}H]CGP-12177 binding, ICI 89,406 competed for only two sites; the highest affinity site had a K_i of 1.02 ± 0.13 nM and a B_{max} of 4.49 ± 0.21 fmol/mg of tissue and the second site had a K_i of 117.9 ± 17.5 nM and a B_{max} of 0.92 ± 0.10 fmol/mg of tissue. In these analyses, the K_d values of [^{3}H]DHA or [^{3}H]CGP-12177 for β_1- and β_2-adrenergic receptors were set equal to each other. In saturation studies of [^{3}H]CGP-12177 binding in cerebral cortex versus cerebellum, the K_d was 0.11 ± 0.003 nM in the cortex versus 0.162 ± 0.008 nM in the cerebellum ($p < 0.05$). Conversely, saturations of [^{3}H]DHA binding (with nonspecific binding defined by LIGAND) yielded K_d values in the cortex of 0.60 ± 0.05 nM versus 0.32 ± 0.05 ($p < 0.05$) in the cerebellum. These data indicate some subtype selectivity for both ^{3}H-ligands, which probably explains the nonequality of the densities of β_1- and β_2-adrenergic receptors determined with the radioligands and ICI 89,406.

Discussion

[^{3}H]DHA has been used to characterize CNS β-adrenergic receptors in numerous studies (24). Nonspecific binding of [^{3}H]DHA has been defined utilizing high (1–100 μM) concentrations of the β-adrenergic antagonists propranolol or alprenolol or the agonist isoproterenol. The accurate quantification of β-adrenergic receptor characteristics is dependent on the selectivity of both the radioligand, [^{3}H]DHA, and the unlabeled competing drug used to define nonspecific binding. Although Scatchard analysis of "specific" [^{3}H]DHA binding, as defined by these drugs, yields linear plots suggestive of selective β-adrenergic receptor labeling by [^{3}H]DHA, the results of the present paper clearly demonstrate that [^{3}H]DHA is not a selective ligand for β-adrenergic receptors. Our data suggest that both [^{3}H]DHA

TABLE 2

Nonlinear regression analysis (LIGAND) of the competition curves of various serotonergic agonists and antagonists for [^{3}H]DHA and [^{3}H]CGP-12177 binding in rat cerebral cortex membranes

The values in the table are the result of at least three competition curves analyzed by LIGAND. In computer analysis of the competition curves, the K_d of [^{3}H]DHA for the unknown site was set at 6.64 nM (see Fig. 1) while the K_d for the β-adrenergic receptor site was set at 0.60 nM. ND indicates that only one site was detected in the competition curve analysis.

Drug	5HT receptor specificity	[^{3}H]DHA binding		[^{3}H]CGP-12177 binding
		Unknown site K_i	β-adrenergic receptor K_i	β-adrenergic receptor K_i
			nM	
8-OH-DPAT	1A	ND	20,806 ± 7,000	>10,000
Spiperone	1A, 2	15,229 ± 4,380	557 ± 176	
RU24969	1A, 1B	29.4 ± 9.0	756 ± 92.	564 ± 160
CGS-12066B	1B	38.4 ± 14.2	576 ± 18	649 ± 205
Mesulergine	1C	ND	7,172 ± 2,597	>10,000
Metergoline	1, 2	48.32 ± 11.87	2,348 ± 148	
Ketanserin	2	ND	17,071 ± 3,928	>10,000
Quipazine	3	ND	2,006 ± 507	

and the drugs used to define its nonspecific binding all bind to a second site. This can lead to an overestimation of β-adrenergic receptors of nearly 2-fold.

Visual inspection of the competition curves for alprenolol, propranolol, or isoproterenol on [^{3}H]DHA binding (using a concentration of [^{3}H]DHA close to its apparent affinity for β-adrenergic receptors) all demonstrated that the competition curves continued to decrease with no leveling off at the concentrations of these drugs typically used to define nonspecific binding. This observation immediately demonstrates that these drugs cannot be used to define nonspecific binding and, thus, the parameters of [^{3}H]DHA binding to β-adrenergic receptors will not be determined accurately by typical methods such as Scatchard analysis.

One may argue that a lower concentration of alprenolol or propranolol would be "safe" and provide an accurate determination of β-adrenergic receptors. However, this suggestion can be criticized for two reasons. 1) If both radioligand and displacing drug bind to two distinct sites, albeit with different affinities, there will always be contamination of one site by the other, unless the displacing drug has such marked differences in affinities for the two sites that its competition curve shows a clear plateau (of at least 1 log unit), allowing for selective displacement of the radioligand from only one of its binding sites. This criterion is not met by either alprenolol or propranolol. 2) In using nonselective drugs, a great risk is run when the system is manipulated via, for example, pharmacological or surgical methods. A change in the affinities of either radioligand or displacing drug for one or both of their binding sites would invalidate the choice of the particular concentration of drug used to define specific receptor binding in the control tissue.

The nonlinear, iterative, curve-fitting program LIGAND (16) has the advantage that it can analyze the total binding of a radioligand according to the law of mass action, with no assumptions being necessary as to the pharmacological characteristics of the binding sites that are being labeled. Saturation data of "total" binding can be analyzed by the program without information as to what was pharmacologically defined as specific versus nonspecific binding, by fitting the data points to the theoretical curves that would be generated by the binding of the radioligand to one, two, or more saturable sites plus nonspecific binding, which is linearly proportional to the concentration of the radioligand. Such an analysis demonstrated that [^{3}H]DHA binds to a single saturable site with a B_{max} (5.42 ± 0.19 fmol/mg of tissue) that was significantly lower than that obtained using Scatchard analysis of specific binding defined by the commonly used competing ligands (Table 1). Indeed, even LIGAND analysis of specific binding defined by these drugs gave a similar erroneous result. Alprenolol-defined specific binding was fitted best to a single site (B_{max} = 9.61 ± 0.33 fmol/mg of tissue) and LIGAND could not fit the specific binding data to two saturable sites over the concentration range of [^{3}H]DHA typically used in such studies. Scatchard plots of such data, however, gave a visual indication that the plots were not linear and, if the concentrations of [^{3}H]DHA used were increased, the plots became more curvilinear, with a linear fit producing increasing B_{max} values as the highest concentration of [^{3}H]DHA used in the saturation experiments was increased. It is of interest to note that, even though the Scatchard plots were clearly curvilinear, the correlation coefficients for a linear plot were still very high ($r > 0.87$). However, even under these circumstances of high [^{3}H]DHA concentrations, LIGAND could not fit specific binding to more than one site. It was apparent that the K_d of [^{3}H]DHA for its second binding site must be appreciably higher than the K_d for β-adrenergic receptors but, because these sites did not appear to saturate, LIGAND could not accurately quantify them. The binding to this second site was included within nonspecific binding by the analysis because, at these high concentrations of [^{3}H]DHA, the component of binding to the second site was small relative to the high levels of noisy nonspecific binding.

Although LIGAND could not characterize the parameters of the second site directly from the [^{3}H]DHA saturation experiments, it was clear from the alprenolol competition curves for [^{3}H]DHA binding that appreciable binding of [^{3}H]DHA and alprenolol was occurring to this second site. We used LIGAND analysis of the alprenolol competition curves for [^{3}H]DHA binding to estimate the binding parameters of this site. The K_d of [^{3}H]DHA for the second site was determined to be approximately 6.64 ± 0.71 nM. Its B_{max} was about 6.47 ± 0.53 fmol/mg of tissue, within the general range of typical CNS neurotransmitter receptor sites.

These approximate parameters for the second site were useful in further characterizing the pharmacological specificity of this site. Because other studies have suggested that β-adrenergic ligands often label serotonin receptors (12, 13) and [^{3}H]DHA binding has been shown recently to be sensitive to serotonin (20), we investigated the competition of selective serotonin receptor subtype agonists and antagonists for [^{3}H]DHA binding. Although the majority of such drugs ($5HT_{1A}$, $5HT_{1C}$, $5HT_2$, and $5HT_3$ receptor subtype selective) only competed for [^{3}H]DHA binding at high concentrations, two drugs, RU24969 and CGS12066B, demonstrated about 20% competition at nanomolar concentrations (Fig. 8). Using the affinity determined by LIGAND from the alprenolol competition curves for the second site to analyze these $5HT_{1B}$ receptor-selective drug competition curves, we determined K_i values for these drugs at the second site to be on the order of 10–50 nM, similar to their affinities for $5HT_{1B}$ receptor sites. Metergoline, a potent antagonist at all serotonergic receptors, also showed a competition curve that best fit a two-site model, with the K_i for the highest affinity site being 48 nM, close to its reported affinity for $5HT_{1B}$ receptors (25). This again suggested that the second site labeled by [^{3}H]DHA could be a serotonergic binding site. The K_i values of RU24969 at the $5HT_{1B}$ receptor binding site that other authors have reported are lower than those we found for the second site labeled by [^{3}H]DHA (25, 26). This could be due to differences in buffer compositions, because receptor/agonist interaction could be influenced by the presence of ions such as Mg^{2+}. However, we cannot conclusively determine whether [^{3}H]DHA is binding to $5HT_{1B}$ receptors because the "binding signal" of [^{3}H]DHA at this unknown site is very poor, mitigating against its accurate characterization.

Isoproterenol also suffered from problems similar to those of alprenolol and propranolol when it was used to define specific β-adrenergic receptor binding. It also competed for [^{3}H]DHA binding with two affinity components. Because isoproterenol is an agonist, it is unclear whether the high and low affinity binding sites represent high and low affinity conformations of the β-adrenergic receptor for the agonist or whether they represent two distinct binding sites on different cellular components. LIGAND analysis suggests, from the similarity in B_{max}

values, that the high affinity site is indeed β-adrenergic receptor binding and that the lower affinity site is the non-β-adrenergic receptor site. This hypothesis is somewhat reinforced by the finding that GTP causes a small shift in the affinity of isoproterenol for its highest affinity site (as might be expected for the adenylate cyclase-linked β-adrenergic receptor), whereas, in contrast, it completely obliterated the low affinity competition of isoproterenol for [^{3}H]DHA binding. This suggests that the second binding site for [^{3}H]DHA is also associated with a GTP-binding protein. If 10 μM isoproterenol was used to define specific [^{3}H]DHA binding to β-adrenergic receptors, a B_{max} was obtained by Scatchard and LIGAND analysis that was intermediate between that defined by LIGAND analysis of total binding or LIGAND analysis using specific binding defined by alprenolol (Table 1). This suggests that studies that have used isoproterenol at this concentration may more closely approximate true β-adrenergic receptor characteristics. However, because the isoproterenol competition curve does not level off and because agonist binding is typically sensitive to many modulators such as the buffer ionic composition, pH, etc., as well as guanine nucleotides, data obtained using it to quantify receptor B_{max} are open to question.

In contrast to its competition for [^{3}H]DHA binding, alprenolol had a monophasic competition for the binding of the other β-adrenergic receptor antagonist, [^{3}H]CGP-12177, and the competition curve leveled off between 0.1 and 100 μM. These data suggest that [^{3}H]CGP-12177 must be a more selective β-adrenergic receptor radioligand. Saturation data for [^{3}H]CGP-12177, whether analyzed by Scatchard or LIGAND analysis of specific binding as defined by alprenolol or by LIGAND analysis of total binding, yielded comparable B_{max} values, reinforcing this conclusion. Indeed, the $5HT_{1B}$ receptor-selective drugs did not compete for [^{3}H]CGP-12177 binding at concentrations lower than 100 nM. Also, the B_{max} calculated by the LIGAND analysis of total [^{3}H]DHA binding was close to the B_{max} calculated by LIGAND for specific [^{3}H]CGP-12177 binding. Isoproterenol competed in a monophasic way for [^{3}H]CGP-12177 binding and the presence of GTP in the incubation mixture significantly shifted the curves to the right, by a factor of 3. Because in the presence of GTP the [^{3}H]CGP-12177 binding sites are homogeneous with respect to agonist affinity, we interpret these results to suggest that, in our assay system (in the absence of divalent cations), the β-adrenergic receptors are not in a high affinity agonist binding state and GTP has a significant, although small, effect on the low affinity agonist binding state of the receptor. However, when 1 mM EDTA was added during membrane preparation and 2.5 mM Mg^{2+} was added in the incubation mixture (27), 60% of isoproterenol competition for [^{3}H]CGP-12177 is of higher affinity (K_i = 35 nM) and is sensitive to GTP (data not shown).

It is of interest that the LIGAND analysis of total binding of [^{3}H]CGP-12177 yielded a slightly, but significantly, lower B_{max} than the LIGAND analysis of alprenolol-defined specific [^{3}H]CGP-12177 binding. This observation suggests that, although [^{3}H]CGP-12177 is a far more selective β-adrenergic receptor ligand than is [^{3}H]DHA, a small component of its binding may occur to another site. However, this is argued against by the fact that the alprenolol competition curve can only be resolved into a single site by LIGAND. Competition curves of nonradioactive CGP-12177 for [^{3}H]DHA binding clarify this observation. CGP-12177 competes for [^{3}H]DHA binding with three components. Two components have high affinity (K_i = 0.13 and 9.41 nM). The highest affinity component has a K_i and B_{max} identical to those determined from the direct binding of [^{3}H]CGP-12177 in saturation studies. The second component has a B_{max} that is identical to the difference between the B_{max} of [^{3}H]CGP-12177 binding and the B_{max} for [^{3}H]DHA binding, determined from their total binding by LIGAND. This suggests that [^{3}H]CGP-12177 labels only a component (approximately 80%) of the β-adrenergic receptors that are labeled by [^{3}H]DHA. Two possibilities could explain this observation. 1) Because the subtype selectivities of [^{3}H]CGP-12177 and [^{3}H]DHA for β_1- and β_2-adrenergic receptors are different, these two sites could represent β_1- and β_2-adrenergic receptors. However, the relative proportion of β_1 to β_2 receptors in the cortex is approximately 4 to 1. Furthermore, [^{3}H]DHA has a selectivity for β_2 receptors over β_1 receptors of about 2.3-fold (8). It is known that [^{3}H]CGP-12177 has a selectivity for β_1 receptors in non-CNS tissue of approximately 2.7-fold (28). Our experiments comparing cortex with cerebellum binding of [^{3}H]CGP-12177 suggest that its selectivity for β_1- versus β_2-adrenergic receptors is less than a factor of 2 in the CNS. Thus, the two highest affinity components of CGP-12177 competition for [^{3}H]DHA binding, with K_i values of 0.13 and 9.41 nM, cannot represent the differential affinities of CGP-12177 for β_1- and β_2-adrenergic receptors. 2) CGP-12177 is known to be very hydrophilic, whereas [^{3}H]DHA is lipophilic. This suggests that the lower affinity component of CGP-12177 competition for [^{3}H]DHA binding may represent an "apparent" lower affinity binding site caused by the lower accessibility of CGP-12177 for receptor sites within the lipid environment of the membrane that are easily accessible to [^{3}H]DHA. This 9.41 nM affinity site of CGP-12177 is not the serotonergic site, because its B_{max} is much lower than that observed for [^{3}H]DHA binding to the putative serotonergic site and the serotonergic $5HT_{1B}$ drugs do not compete for any [^{3}H]CGP-12177 binding at concentrations less than 100 nM.

In conclusion, [^{3}H]DHA is not a satisfactory ligand for the characterization of β-adrenergic receptors if nonspecific binding is defined using classical agonists or antagonists. [^{3}H]DHA can be used to quantify β-adrenergic receptors if its total binding is analyzed by the computer program LIGAND or if selective β-adrenergic receptor antagonists are used to define nonspecific binding. This is not simply a methodological issue, because we show in the accompanying paper that the failure to recognize the presence of this non-β-adrenergic component of [^{3}H]DHA binding and its independent regulation has led to the misinterpretation of data relating to the mechanism of action of antidepressant drugs and the permissive role that serotonergic systems play in the regulation of β-adrenergic receptor number.

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