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[^{3}H]DIHYDROERGOCRYPTINE BINDING TO BOVINE STRIATAL MEMBRANES DEFINED BY A LOW *d*-BUTACLAMOL CONCENTRATION

ANTAGONISM BY SUBSTITUTED BENZAMIDES

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Abstract—An apparent dopamine-D2 receptor binding system was characterized using [^{3}H]dihydroergocryptine (DHE) as the ligand. Specific binding to bovine striatal membranes was defined as the difference between binding in the absence and in the presence of 4 nM *d*-butaclamol. These high affinity binding sites (K_d =0.24 nM) were saturable (B_{max} = 75 fmoles/mg protein), apparently homogeneous, non-interacting, and stereospecific. Specific binding was greatest in areas rich in dopaminergic innervation. In competition experiments, apomorphine (IC_{50} = 100 nM) was fifty times more potent than dopamine which, in turn, was nine times more potent than *l*-norepinephrine and serotonin. Antipsychotic drugs including the substituted benzamides—metoclopramide, sulpiride and sultopride—were active in competing with [^{3}H]DHE for these binding sites (IC_{50} = 100 nM), and their affinities agreed well with their anti-dopaminergic potencies *in vivo*. Potencies of antipsychotic drugs in competing for DHE binding sites were increased compared to their potencies in competing for [^{3}H]spiroperidol binding sites using 1 μM *d*-butaclamol as the masking ligand. This relative increase was about 10-fold greater for the substituted benzamides than for other antipsychotic drugs. The relative inactivity of substituted benzamides in competing for binding sites labeled by spiroperidol was accounted for by their greater selectivity for dopamine receptors. In binding studies using a non-selective radioligand, selectivity could be enhanced by defining specific binding sites with a low concentration of masking ligand.

All neuroleptic drugs with proven clinical efficacy are dopamine (DA) receptor antagonists. They increase DA turnover *in vivo* by a mechanism presumed to be secondary to DA receptor blockade [1, 2]. This effect can be monitored conveniently by measuring the dose-dependent increase in levels of 3,4-dihydroxyphenylacetic acid (DOPAC), the major DA metabolite in rat striatum [3, 4], evoked by these drugs. The pharmacological and clinical potencies of most of these drugs correlate with their abilities to compete with [^{3}H]butyrophenones for binding sites in striatal membrane preparations [5–7]. These sites are believed to represent DA receptors. For one class of antipsychotic drugs, the substituted benzamides, the correlation is poor. Substituted benzamides such as metoclopramide and sulpiride antagonize DAergic systems *in vivo* [8–11] but are relatively inactive in the binding assays [12–15]. Moreover, although other classes of antipsychotic drugs antagonize the stimulation of adenylate cyclase by DA [16, 17], the benzamides are inactive [8, 18, 19]. These apparently contradictory findings regarding the properties *in vivo* and *in vitro* of substituted benzamides have aroused speculation as to the mechanism of action of these drugs [13, 20].

Based on their functional linkage with adenylate cyclase activity, DA receptors have been classified into two types. DA receptors that are linked to adenylate cyclase are termed D1-receptors, and DA receptors that are independent of cyclase activity are termed D2-receptors [21]. *cis*-Flupenthixol and dihydroergocryptine (DHE) have been proposed as radioligands for the study of D1- and D2-receptors respectively. DHE is an ergot derivative, and ergots related to DHE decrease DA turnover [22, 23], inhibit prolactin secretion [24], and elicit a behavioral stereotypy in animals [22, 25]; unlike DA, however, they are relatively inactive in stimulating adenylate cyclase [26–29]. This property of influencing DAergic function without affecting adenylate cyclase activity is analogous to the benzamide antipsychotics. The pharmacological effects of ergots expressed through DA systems may predominantly involve the D2-receptors. In fact, DHE and metoclopramide have been taken as a D2-receptor agonist–antagonist pair [21]. It was of interest, therefore, to delineate a binding system with DAergic properties, using [^{3}H]DHE as the radioligand. This system might label a subclass of DA receptors at which the benzamides exert their anti-DA effects *in vivo*. Accordingly, we studied [^{3}H]DHE binding to bovine brain tissue. DHE has been used recently to identify DAergic binding sites in striatum and anterior pituitary [24, 30]. Since it has also been used previously as a ligand for α-noradrenergic binding sites [31, 32], we investigated the possibility of enhancing the selectivity of the system by reducing the concentration of masking drug used in defining specific binding of

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[^{3}H]DHE. We report here the properties of DHE binding sites sensitive to 4 nM *d*-butaclamol, the regional distribution of the binding sites, and the interaction of substituted benzamides and other classes of antipsychotic drugs toward these sites. We also report on differences between this binding system and the binding of [^{3}H]spiroperidol in the presence of 1 μM *d*-butaclamol.

MATERIALS AND METHODS

Fresh bovine brains were obtained on ice from a slaughterhouse. Striata and other regions were dissected, removed and frozen in a −80° freezer for periods of up to 2 weeks. [^{3}H]DHE (sp. act. 30.9 Ci/mmole) and Formula-963 scintillation mixture were purchased from the New England Nuclear Corp., Boston, MA. The purity (> 95 per cent) of [^{3}H]DHE was checked by two thin-layer chromatography systems [chloroform–toluene–ethanol–ammonium hydroxide (4:2:1:0.1); chloroform–ethanol–glacial acetic acid (9:5:1)]. Whatman glass fiber GF/C filters and ascorbic acid were purchased from the Fisher Scientific Co., Springfield, NH. The drugs were supplied by the following companies: DHE, clozapine, perlapine, and thiethylperazine (Sandoz, East Hanover, NJ); lergotrile (Eli Lilly, Indianapolis, IN); loxapine (Lederle, Pearl River, NY); sulpiride (Warren-Teed, Horsham, PA); metoclopramide (A. H. Robbins, Richmond, VA); spiroperidol (SPIRO); domperidone, and penfluridol (Janssen, Beerse, Belgium); tiapride and sultopride (DeLagrange, Paris, France); chlorpromazine (Smith, Kline & French, Philadelphia, PA); *d*- and *l*-butaclamol (Ayerst, Montreal, Canada); fluphenazine (E. R. Squibb, Princeton, NJ); U-25,927 (The Upjohn Co. Kalamazoo, MI); haloperidol (McNeil, Fort Washington, PA); *cis*- and *trans*-flupenthixol (H. Lundbeck, Copenhagen, Denmark); pargyline (Abbott Laboratories, North Chicago, IL); phentolamine (Ciba Geigy, Summit, NJ); and apomorphine (Merck & Co., Inc., Rahway, NJ). Serotonin (5-HT), *l*-norepinephrine [(−)NE], procainamide, methysergide, and bovine serum albumin were purchased from the Sigma Chemical Co., St. Louis, MO.

Tissues were prepared as described [13]. Bovine caudate and other regions were homogenized in 50 vol. of ice-cold 50 mM Tris–HCl buffer (pH 7.7) at 25°, with a Brinkman polytron PT-10 (setting 3, 15 sec). The homogenate was centrifuged at 1000 *g* for 10 min (Sorvall RC 2B, rotor SS-34). The pellet (P1) was discarded and the supernatant fraction (S1) was centrifuged at 39,000 *g* for 10 min. The 39,000 *g* pellet (P2) was washed and rehomogenized with a Teflon–glass homogenizer (setting 7, 15 up and down strokes) in fresh Tris–HCl buffer and recentrifuged at the same speed. The final pellet was then rewashed and rehomogenized at a final concentration of 20 mg of original wet weight per ml in a cold, fresh 50 mM Tris-HCl buffer containing 0.2% ascorbic acid, 20 μM pargyline, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.1, at 37°. This final suspension was placed in a 37° bath for 5 min and returned to ice.

In saturation experiments, 100 μl of increasing concentrations of [^{3}H]DHE diluted with 0.1% ascorbic acid, 100 μl of 0.1% ascorbic acid or *d*-butaclamol (final concentration 4×10^{-9} M), and 800 μl of tissue suspension were added to each tube. The range of [^{3}H]DHE used was 0.05 to 2.5 nM. All determinations were done in triplicate.

In competition experiments, 100 μl of [^{3}H]DHE (final concentration 0.25 nM), 50 μl of increasing concentrations of competing drugs diluted in 0.1% ascorbic acid, 50 μl of 0.1% ascorbic acid or *d*-butaclamol solution (final concentration 4 nM), and 800 μl of tissue suspension were added to each tube.

All tubes were incubated under dim light at 23° for 100 min and rapidly filtered under vacuum through Whatman glass fiber GF/C filters. After three 5-ml rinses with ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 23°), the filters were placed in glass scintillation vials with 9 ml of Formula-963 scintillation mixture shaken in the dark for 15 min, and analyzed by liquid scintillation spectrometry (Beckman LS-9000) at 43 per cent counting efficiency. All counts were converted to dpm.

There was no apparent degradation [^{3}H]DHE during the incubation procedure, as chromatograms of the labeled compound were similar prior to and after the incubation period.

Saturable or specific binding was defined as the difference between binding in the absence and in the presence of 4 nM *d*-butaclamol. This low concentration of *d*-butaclamol was selected to define apparent DA sites for the following reasons. First, DHE has been shown to possess high affinity for α-noradrenergic, 5-HT, and DA sites (Table 1). Therefore, at the nM range, [^{3}H]DHE would label more than one receptor site. Second, *d*-butaclamol interacts with DA sites in the nM range, and interacts with α-noradrenergic and 5-HT sites in the 10^{-8} M range (Table 1). At a concentration greater than 10^{-8} M, *d*-butaclamol would certainly bind to more than one receptor population. Therefore, to define a subclass of DA sites within the multiple [^{3}H]DHE binding

Table 1. Affinity of spiroperidol, *d*-butaclamol, and DHE for various binding sites

Compound	Radioligand	Putative receptor	K_i (nM)
Spiroperidol	Haloperidol	DA	0.2*
	WB-4101	α-NE	18†
	LSD	5-HT	6.3‡
d-Butaclamol	Haloperidol	DA	0.54*
	WB-4101	α-NE	24†
	LSD	5-HT	42‡
DHE	DHE	DA	0.54§
	DHE	α-NE	1.55‖
	LSD	5-HT	7.5¶

* D. R. Burt, I. Creese and S. H. Snyder, *Molec. Pharmac.* **12**, 800 (1976).

† S. J. Peroutka, D. C. U'Prichard, D. A. Greenberg and S. H. Snyder, *Neuropharmacology* **16**, 549 (1977).

‡ Ref. 7.

§ Ref. 30.

‖ M. I. Holck, B. H. Marks and C. A. Wilberding, *Molec. Pharmac.* **15**, 77 (1979).

¶ D. Greenberg and S. H. Snyder, *Life Sci.* **20**, 927 (1977).

sites, it is necessary to lower the concentration of *d*-butaclamol. Based on its affinity for various receptor sites, 4 nM *d*-butaclamol would preferentially bind to the DA sites with minimal interaction with the α-noradrenergic or 5-HT sites. As shown below, although this concentration of *d*-butaclamol seems to underestimate the number of binding sites in the saturation experiment, experimentally this error does not greatly affect the calculated values of the dissociation constant (K_d) and the total receptor density (B_{max}) for [^{3}H]DHE.

The displacement of [^{3}H]DHE binding by *d*-butaclamol was studied. As shown in Fig. 1, the suppression of [^{3}H]DHE binding required more than five log concentration units of *d*-butaclamol, and a 50 per cent inhibition occurred at 4 nM. There was no apparent plateau over the entire range studied (10^{-10} to 10^{-5} M), and it was not possible to select a concentration of *d*-butaclamol that would appear to suppress only one component of binding sites labeled by [^{3}H]DHE. The slope of the displacement curve of [^{3}H]DHE binding by *d*-butaclamol in the nM range however, was steeper than at higher concentrations. Since *d*-butaclamol in the nM range was so effective in competing against [^{3}H]DHE binding, and since *d*-butaclamol in the nM range shows selectivity for DAergic sites (Table 1), we decided to investigate the properties of [^{3}H]DHE binding sites using 4 nM *d*-butaclamol as the masking drug.

Based on the affinity of *d*-butaclamol for the 4 nM *d*-butaclamol sensitive [^{3}H]DHE sites given in Table 2, the per cent receptor occupancy by 4 nM *d*-butaclamol in the presence of 0.25 nM [^{3}H]DHE, the concentration used in the competition experiments, is:

$$B/B_m = L/[L + K_m(1 + I/K_i)] \qquad (1)$$

$$= 4/[4 + 0.78(1 + 0.25/0.25)] = 72\%.$$

In the saturation experiments, the highest concentration of [^{3}H]DHE used was 2.5 nM, and 4 nM *d*-butaclamol could theoretically occupy only 32 per cent of these sites (Eqn. 1). It would seem, therefore, that the B_{max} of [^{3}H]DHE sites, defined as the difference in binding in the absence or presence of 4 nM *d*-butaclamol, is underestimated and that K_d values are also affected by this error. The validity of using 4 nM *d*-butaclamol as the masking drug, however, is supported by the following experiments. First, a saturation experiment was performed using 20 nM *d*-butaclamol to define non-specific binding. At this concentration *d*-butaclamol occupies 70 per cent of the receptor sites when [^{3}H]DHE is at 2.5 nM, and 93 per cent when [^{3}H]DHE is 0.25 nM (Eqn. 1). Under these conditions the K_d was 0.2 nM, the B_m was 60 fmoles/mg protein, the Scatchard plot was linear, and the Hill coefficient was 0.93. These values do not differ significantly from the K_d (0.25 nM), B_m (75 fmoles/mg protein), linear Scatchard, and unity Hill coefficient obtained with 4 nM *d*-butaclamol (see Results). Second, a saturation experiment was performed using various *d*-butaclamol concentrations as masking ligand to define specific binding. The concentrations were chosen to provide a fixed percentage of receptor sites occupied (72 per cent) at various [^{3}H]DHE concentrations. A linear Scatchard plot and unity Hill coefficient were observed. The K_d (0.4 nM) and B_m (80 fmoles/mg protein) values again did not vary significantly from results obtained with 4 nM *d*-butaclamol. Third, we have reanalyzed the saturation experiments done with 4 nM *d*-butaclamol by eliminating points with high [^{3}H]DHE concentrations, as these points were supposed to contain greater errors. We found that the K_d and B_m were virtually unchanged. Thus, although 4 nM *d*-butaclamol may have underestimated the number of binding sites, experimentally these errors do not seem to affect greatly the calculated values of K_d and the B_{max} of [^{3}H]DHE and the apparent homogeneity of the binding sites.

In competition experiments it can be shown that, in the presence of the competitor, the specific binding, defined as the difference between binding in the absence and presence of 4 nM *d*-butaclamol, is:

total binding − non-specific binding =

$$\frac{1}{1 + K_d/D(1 + A/K_a)} - \frac{1}{1 + K_d/D(1 + A/K_a + B/K_b)}, \qquad (2)$$

where D and K_d are the concentration and dissociation constant of [^{3}H]DHE, A and K_a are the concentration and dissociation constant of the competitor, and B and K_b are the concentration and dissociation constant of *d*-butaclamol. The amount of specific binding for [^{3}H]DHE in the absence of competitor is:

total binding − non-specific binding =

$$\frac{1}{1 + K_d/D} - \frac{1}{1 + K_d/D(1 + B/K_b)}. \qquad (3)$$

The amount bound by the competitor is, therefore, (3)–(2)

$$= \frac{1}{1 + K_d/D} - \frac{1}{1 + K_d/D(1 + B/K_b)}$$

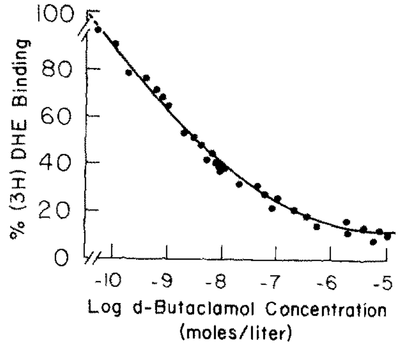

Fig. 1. Inhibition of [^{3}H]DHE binding by *d*-butaclamol. Triplicate test tubes containing tissue homogenate, [^{3}H]DHE (0.23 nM), and increasing concentrations of *d*-butaclamol were incubated at 23° for 100 min. The radioactivity in the absence of *d*-butaclamol (100 per cent) was 3200 dpm. Each point on the figure is the average of three samples in one experiment. The standard deviation for each point was within the range of the symbol.

$$-\frac{1}{1+K_d/D(1+A/K_a)}+\frac{1}{1+K_d/D(1+A/K_a+B/K_b)} \quad (4)$$

When $D = K_d$, $B \gg K_b$, the amount bound by the competitor when $A = K_a$ is

$$\frac{1}{1+1/1}-\frac{1}{1+1(1+\infty)}-\frac{1}{1+1(1+1)}+\frac{1}{1+1(1+1+\infty)}=1/2-1/3$$
$$=1/6=0.167. \quad \text{(Eqn.4)}$$

When $D = K_d$, $B \gg K_b$, the amount bound by the competitor when $A = 100\,K_a$ is

$$\frac{1}{1+1/1}-\frac{1}{1+1(1+\infty)}-\frac{1}{1+1(1+100)}+\frac{1}{1+1(1+100+\infty)}$$
$$=1/2-1/102=0.49. \quad \text{(Eqn.4)}$$

In our experiments when $D = K_d$, $B = 4$, and $K_b =$ $= 0.78$, the amount bound by the competitor when $A = K_a$ is

$$\frac{1}{1+1}-\frac{1}{1+(1+4/0.78)}-\frac{1}{1+1(1+1)}+\frac{1}{1+1(1+1+4/0.78)}$$
$$=1/2-1/7.13-1/3+1/8.13=0.147.$$

When $D = K_d$, $B = 4$, $K_b = 0.78$, the amount bound by the competitor when $A = 100\,K_a$ is

$$\frac{1}{1+1}-\frac{1}{1+(1+4/0.78)}-\frac{1}{1+1(1+1000)}+\frac{1}{1+1(1+100+4/0.78)}$$
$$=1/2-1/7.13-1/102+1/107.13=0.3592.$$

The magnitude of error when $A = K_a$ is, therefore, $(0.147 - 0.167)/0.167 = -0.12 = 12\%$. The magnitude of error when $A = 100\,K_a$ is $(0.3592 - 0.49)/0.49 = -0.27 = 27\%$. Thus, the magnitude of error introduced is 12–27% when the range of the concentration of the competitor increases from 1 K_a to 100 K_a. This amount of error is not sufficient to invalidate the measurement of K_i for various competitors when specific [^{3}H]DHE binding is defined with 4 nM *d*-butaclamol as masking drug.

Data from binding experiments were analyzed by the PROPHET computer of the National Institutes of Health. Based on the Michaelis–Menten equation, a computerized procedure calculates the dissociation constant (K_d) of [^{3}H]DHE and the total receptor density (B_{max}). The inhibition constants (K_i) for drugs showing unity Hill coefficients were analyzed by the Dixon plot [33]. IC$_{50}$ Values for all drugs were computed based on the Hill analysis.

Protein concentrations were measured by the method of Lowry *et al.* [34] with crystalline bovine serum albumin as the standard.

RESULTS

Our results showed that the specific binding of [^{3}H]DHE to membrane preparations from bovine striatum, defined as the difference between binding in the absence and presence of 4 nM *d*-butaclamol, was apparently homogeneous, reversible, stereospecific, and heat sensitive. In saturation experiments, specific binding reached a plateau at approximately 0.5 nM [^{3}H]DHE (Fig. 2) after a 100-min incubation period at 23°. The B_{max} and the K_d, determined by the hyperbolic fit, were 0.075 ± 0.006 pmole/mg protein and 0.244 ± 0.06 nM, respectively, for an average of five determinations. In each determination, the Scatchard plot was linear ($R > 0.91$) (Fig. 3), and the Hill analysis did not show any indication of multiple binding sites or cooperativity (Hill coefficient $= 0.98 \pm 0.04$). Thus, despite its high affinity for various receptor types, [^{3}H]DHE binding sites defined with a low *d*-butaclamol concentration appeared to be homogeneous. It should be noted that the non-specific binding, or binding in the presence of 4 nM *d*-butaclamol, was non-linear in four out of five cases. At 0.24 nM, the concentration used in the competition experiments, specific binding was 45–55 per cent of the total binding.

The kinetics of [^{3}H]DHE binding was carried out at a radioligand concentration of 0.16 nM at 23°. In the association experiment, there was no specific binding in the first 15 min, but after 100 min specific binding had reached a plateau. The level of specific binding at 80 min was 85 per cent of the binding at 100 min. As most of the concentrations employed in the saturation experiments were close to or above 0.16 nM, it was assumed that the system was at equilibrium after a 100-min incubation period. The displacement of [^{3}H]DHE binding was carried out after the association experiment by the addition of 4 nM *d*-butaclamol. Bound [^{3}H]DHE was slowly displaced by 4 nM *d*-butaclamol. A 15 per cent reduction of specific binding was observed after 5 h. [^{3}H]DHE binding, however, was clearly reversible, as 1 μM *d*-butaclamol displaced half of the bound

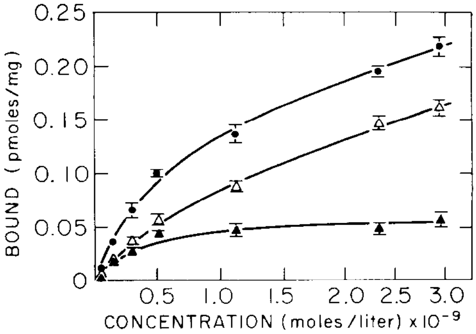

Fig. 2. Saturation of specific [^{3}H]DHE binding sites in bovine caudate membranes. Triplicate tubes containing tissue homogenate (0.7 mg protein/assay) and the indicated amount of [^{3}H]DHE were incubated in 1 ml of incubation medium at 23° for 100 min. Total binding (●) and non-specific binding (△) were determined in the absence and in the presence of 4 nm *d*-butaclamol respectively; the difference between the two is defined as specific binding (▲). Total receptor density (B_{max}) and dissociation constant (K_d) were 0.075 pmole/mg and 0.25 nM respectively. Vertical bars represent standard deviation.

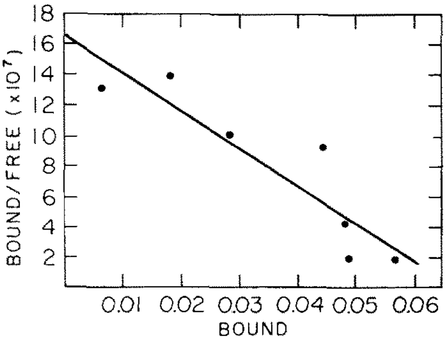

Fig. 3. Scatchard plot of the specific binding in Fig. 2.

[^{3}H]DHE in 1 hr at 30°. Similar slow reversal of binding has been observed for other ligands such as LSD* and QNB (S. Maayani, unpublished; Refs. 35 and 36), and problems with [^{3}H]DHE kinetics have also been reported [32].

Specific binding of [^{3}H]DHE was heat sensitive and linearly dependent on protein concentration. Heating the final homogenate in boiling water for 5 min eliminated specific binding. There was a linear relationship between protein concentration and specific binding up to 1.2 mg protein/tube. In routine experiments, the amount of protein used was 0.7 mg/test tube. There was no specific binding to filters in the absence of tissue. The amount of binding to the filter was 5 per cent of [^{3}H]DHE added, and it was not displaceable by *d*-butaclamol. Specific binding was stable during the experiment.

Stereospecificity of [^{3}H]DHE binding was observed in the competition experiments (Fig. 4): *d*-butaclamol, the clinically active isomer, was 500 times more potent in competing for [^{3}H]DHE sites than the clinically inactive *l*-isomer. *cis*-Flupenthixol, the clinically active thioxanthene, was also more potent than the *trans*-isomer, but the difference in potency was only 8-fold (Table 2).

The regional distribution of [^{3}H]DHE sites was studied using various ligand concentrations. Specific binding in striatum, including part of nucleus accumbens, was 75 fmoles/mg protein. This was three times that of tuberculum olfactorium (23 fmoles/mg protein) and five times that of substantia nigra and frontal cortex (14 fmoles/mg protein). Compared to striatum, only negligible specific binding was detected in medulla (3 per cent), occipital cortex (3 per cent), hippocampus (3 per cent), hypothalamus (6 per cent), and cerebellum (< 1 per cent).

The competition for [^{3}H]DHE binding sites at 0.24 nM [^{3}H]DHE was studied with the agonists DA, apomorphine, 5-HT, and (−)NE (Fig. 5), and with various classes of DA antagonists (Fig. 6 and Table 2). In general, DA antagonists competed effectively for [^{3}H]DHE sites and agonists competed poorly. As shown in Table 2, the IC$_{50}$ values of agonists were in the μM range, whereas the IC$_{50}$ values for the majority of DA antagonists were in the nM range. Among the agonists, the order of potency was compatible with DAergic sites, as apomorphine (IC$_{50}$ = 100 nM) was fifty times more active than DA, which in turn was eight times more potent than (−)NE and 5-HT.

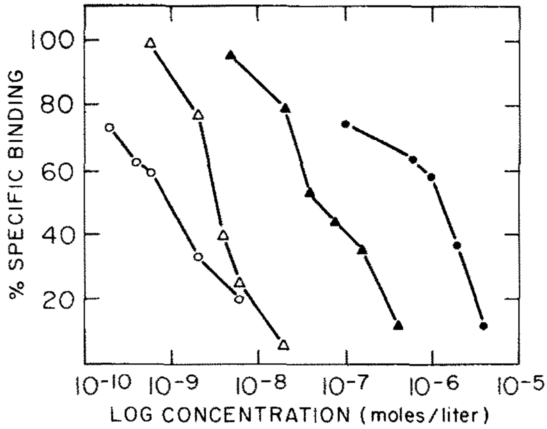

Fig. 4. Effects of some isomers of neuroleptic agents on [^{3}H]DHE sites. *d*-Butaclamol and other drugs were dissolved in a minimal amount of glacial acetic acid and diluted with 0.1 percent ascorbic acid. Specific binding of [^{3}H]DHE at 0.25 nM (K_d) represents 45–55 per cent of the total binding. Incubation conditions are the same as indicated in Fig. 1. Key: *d*-butaclamol (○); *cis*-flupenthixol (△); *trans*-flupenthixol (▲); and *l*-butaclamol (●).

The antipsychotic agents were active in the DHE binding assay and, for the most part, the potencies of drugs within a given class agreed well with their properties *in vivo* (Table 2). For example, the butyrophenone, spiroperidol (IC$_{50}$ = 0.22 nM), was twenty times more active than haloperidol (4.5 nM); the phenothiazine, fluphenazine (2.6 nM), was 2-fold more potent than thiethylperazine (5.9 nM) and four times more potent than chlorpromazine (10 nM); the dibenzoheteroepine, loxapine (15 nM), was twenty times more potent than perlapine (320 nM) or clozapine (320 nM). The most interesting finding was that the benzamides were active in competing for sites labeled by [^{3}H]DHE. The IC$_{50}$ values of metoclopramide, sulpiride, and sultopride were close to 100 nM.

The ergot derivatives DHE, lergotrile, and methysergide competed well with [^{3}H]DHE for binding sites. The inhibition constant of DHE (K_i = 0.44 nM), calculated from the Dixon plot (Fig. 7), agreed well with the K_d of [^{3}H]DHE (0.24 nM) obtained in saturation experiments.

Phentolamine, a strong α-nordrenergic blocker, was weak in the [^{3}H]DHE binding assay, with an IC$_{50}$ in the μM range. This indicates that the [^{3}H]DHE sites defined with 4 nM *d*-butaclamol were not significantly contaminated with α-noradrenergic sites. Procainamide, a benzamide antiarrhythmic agent, was inactive against [^{3}H]DHE sites. At μM concentrations, it displaced only 20 per cent of the specifically bound ligand. It is interesting to note that U-25,927, a clinically inactive butyrophenone that does not increase DA turnover *in vivo* [3], was fairly active in competing with [^{3}H]DHE for binding sites. Although it is much weaker than spiroperidol or haloperidol, it is still stronger than clozapine, a drug with proven antipsychotic efficacy. Domperidone,

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* LSD = D-lysergic acid diethylamide; and QNB = qui- nuclidinyl benzilate.

Table 2. Competition for [^{3}H]DHE sites by drugs

Drugs	K_i* (nM) ± S.E.	(N)	IC$_{50}$† (nM) ± S.E.	(N)	Hill coefficient† ± S.E.	(N)
Agonists						
Apomorphine	62	(2)	98	(2)	0.87	(1)
DA			6,200 ± 1,520	(3)	0.58 ± 0.07	(3)
(−)NE			47,700	(2)	0.64	(2)
5-HT			43,220	(1)	0.63	(1)
Antagonists						
Spiroperidol	0.1	(2)	0.22	(2)	0.94	(2)
Domperidone	0.5 ± 0.12	(3)	1.68 ± 0.75	(3)	0.9	(2)
Haloperidol			4.5 ± 1.02	(3)	0.76	(2)
Fluphenazine	0.49 ± 0.18	(3)	2.59 ± 1.77	(3)	0.94	(2)
Thiethylperazine	2.97 ± 0.67	(3)	5.93 ± 2.15	(3)	1.07	(2)
Chlorpromazine	3.3 ± 0.47	(3)	9.96 ± 1.76	(3)	1.08 ± 0.09	(3)
cis-Flupenthixol	2.43 ± 1.4	(3)	5.87 ± 2.47	(3)	0.85	(2)
trans-Flupenthixol	30 ± 6.8	(3)	43	(2)	0.87	(2)
d-Butaclamol	0.78	(2)	1.26	(2)	0.93	(2)
l-Butaclamol	463	(2)	547	(2)	0.82	(2)
Loxapine	5.91 ± 0.67	(3)	15 ± 1.75	(3)	0.97 ± 0.12	(3)
Clozapine	125	(2)	317	(2)	1.	(1)
Perlapine	306 ± 36	(3)	321	(2)	0.92	(2)
Metoclopramide	44 ± 13	(5)	70 ± 28	(5)	0.90 ± 0.08	(5)
Sultopride	50 ± 12	(5)	91 ± 28	(5)	0.95 ± 0.08	(4)
Sulpiride	99 ± 31	(3)	143 ± 48	(3)	0.81 ± 0.05	(3)
Tiapride			2,908	(2)	0.68	(2)
Ergots						
DHE	0.44 ± 0.22	(3)	0.66 ± 0.13	(3)	1.03 ± 0.015	(3)
Lergotrile			81	(2)	0.62	(2)
Methysergide			231	(1)	1.72	(1)
Others						
Phentolamine			4,000 ± 1,300	(3)	0.75	(2)
U-25,927			324	(2)	1.22	(2)
Procainamide			>20,000	(1)		

* K_i values for drugs with unit Hill coefficients were calculated from the Dixon plot. The concentration of [^{3}H]DHE used was 0.25 nM.

† Value for IC$_{50}$ and Hill coefficients were obtained from Hill plots.

a butyrophenone derivative with anti-emetic properties but which does not cross the blood–brain barrier, was also very active in the present assay.

In Table 3, the IC$_{50}$ values and Hill coefficients of various neuroleptic agents in the [^{3}H]DHE assay were compared with results that we obtained previously in the [^{3}H]SPIRO binding assay, using 1 μM *d*-butaclamol as the masking ligand [13]. These two systems seem to differ in the following respects: (1) except for sulpiride and metoclopramide, the IC$_{50}$

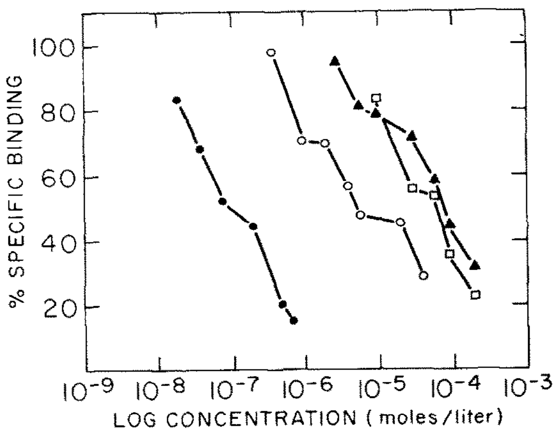

Fig. 5. Effects of various agonists on [^{3}H]DHE sites. Conditions were as indicated in Fig. 1. Key: apomorphine (●); dopamine (○); (—)norepinephrine (▲); and 5-HT (□).

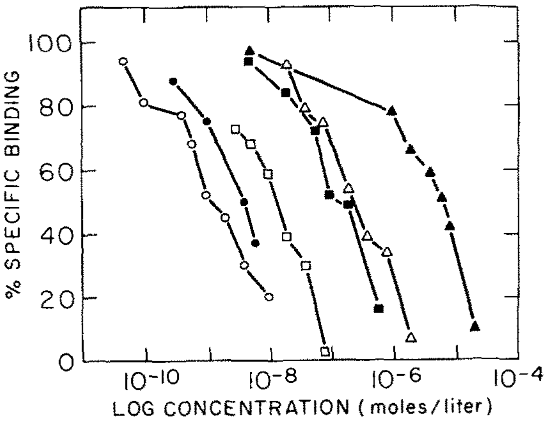

Fig. 6. Effects of DHE, neuroleptics, and phentolamine on [^{3}H]DHE sites. Conditions were as indicated in Fig. 1. Key: DHE (○); haloperidol (●); chlorpromazine (□); metoclopramide (■); sulpiride (△); and phentolamine (▲).

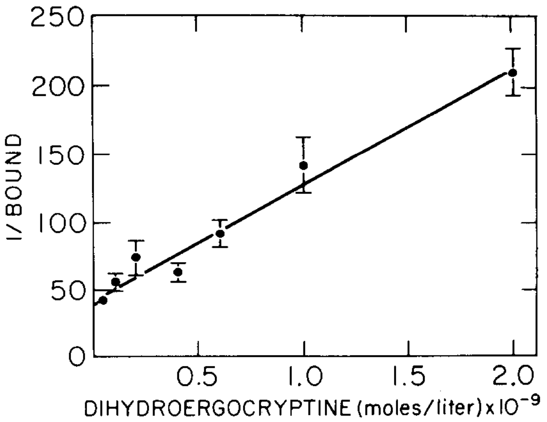

Fig. 7. Dixon plot of unlabeled DHE competing for [^{3}H]DHE sites in bovine caudate membranes. Conditions were as indicated in Fig. 1. The K_i for the unlabeled ligand was calculated from $K_i = K_d/[(B_{max})\ (D^*)] \times 1/(\text{slope})$, where K_i is the inhibition constant of the unlabelled ligand, and K_d and (B_{max}) are the dissociation constant and the total receptor density determined from the saturation experiment (Fig. 2). (D*) is the concentration of [^{3}H]DHE used. Vertical bars represent standard deviation.

values for drugs in the [^{3}H]DHE assay were about one order of magnitude lower than in the [^{3}H]SPIRO assay; (2) the affinity of sulpiride and metoclopramide for [^{3}H]DHE sites was about two orders of magnitude higher than for [^{3}H]SPIRO sites; and (3) judged by the Hill coefficients, an apparent competitive inhibition was generally observed for antipsychotic drugs competing for DHE sites, in contrast to several drugs with low Hill coefficients competing for [^{3}H]SPIRO sites.

In Table 4, the effects of various neuroleptics in competing for [^{3}H]DHE sites, [^{3}H]SPIRO sites and on DOPAC elevation in rat are compared and correlated with the average clinical doses of these compounds. There was a significant correlation between the IC_{50} values for [^{3}H]DHE sites and the clinical doses ($R = 0.86$, $p < 0.002$). A similar correlation existed between the ED_{50} values for DOPAC elevation in rat striatum and the clinical doses ($R = 0.86$, $P < 0.005$). In comparison, a somewhat lower correlation was found between the IC_{50} values for [^{3}H]SPIRO sites and the clinical doses ($R = 0.76$, $P < 0.015$).

DISCUSSION

The characteristics of a population of binding sites are determined by the selectivity and concentration range of both the radioligand and the masking drug utilized. [^{3}H]DHE, the radioligand used in this study, was reported to interact with DA, α-noradrenergic, and 5-HT sites (Table 1). Similarly, *d*-butaclamol, the masking drug selected for this study, also interacts with these sites (Table 1). To enhance the specificity of binding to DAergic sites from the multiple binding sites labeled by [^{3}H]DHE, it was necessary to select a concentration of *d*-butaclamol which would preferentially mask these sites. A concentration of 4 nM *d*-butaclamol was selected for the present study because it selectively interacts with DA sites (Table 1), and it provides a measurable "net" binding, i.e. a sufficient difference between binding in its presence and its absence. As shown in Materials and Methods, although this use of a low concentration of masking drug seems to underestimate the number of binding sites, the results obtained do not differ when the *d*-butaclamol concentration is varied from 4 to 20 nM.

We have demonstrated that specific [^{3}H]DHE binding sites, defined as the difference between binding in the absence and presence of 4 nM *d*-butaclamol, are saturable and stereospecific, and that ligand binding is slowly reversible and heat sensitive. The affinity of apomorphine exceeded that of DA and both had a greater affinity than (−)NE and 5-HT (Table 2). The regional distribution of 4 nM *d*-butaclamol-sensitive [^{3}H]DHE binding sites paralleled the distribution of DA in brain, and the affinity of a variety of DA antagonists, i.e. antipsychotic agents, for these sites agreed well with their phar-

Table 3. Values for IC_{50} and Hill coefficients of neuroleptic agents in the [^{3}H]DHE and [^{3}H]spiroperidol binding assays

	[^{3}H]DHE		[^{3}H]Spiroperidol*		
Drugs	IC_{50} (nM)	Hill coefficient	IC_{50} (nM)	Hill coefficient	IC_{50}(SPIRO) / IC_{50} (DHE)
Spiroperidol	0.22	0.94	3.81	1.01	17
Haloperidol	4.5	0.76	104	0.83	23
Fluphenazine	2.59	0.94	26	1.0	10
Thiethylperazine	5.93	1.07	73	0.98	12
Chlorpromazine	10.	1.08	151	0.92	15
d-Butaclamol	1.26	0.93	4.16	0.74	3
l-Butaclamol	547	0.82	5,290	1.56	10
Loxapine	15	0.97	83	0.72	5.5
Perlapine	321	0.92	3,990	0.51	12
Clozapine	317	1	1,420	0.64	4.5
Metoclopramide	70	0.94	16,900	0.83	241
Sulpiride	143	0.95	29,000	0.87	201
U-25,927	324	1.22	1,429	0.72	44

* Values were taken from Ref. 13. The concentration of [^{3}H]SPIRO was 1 to 1.5 nM; the K_d of [^{3}H]SPIRO was 0.69 nM.

Table 4. Comparison of effects of some neuroleptics on [^{3}H]DHE and [^{3}H]SPIRO sites, on DOPAC elevation in the rat, and to average clinical dose*

Drug	[^{3}H]DHE [IC$_{50}$ ± S.E. (N)]† (nM)	[^{3}H]SPIRO [IC$_{50}$ ± S.E. (N)]‡ (nM)	Striatal DOPAC elevation ED$_{50}$‡ (μmoles/kg)	Average clinical dose‡ (μmoles/kg)
Spiroperidol	0.22 (2)	3.81 (2)	0.4	0.06
d-Butaclamol	1.26 (2)	4.16 ± 0.5 (3)	0.34	2.14
Fluphenazine	2.59 ± 1.77 (3)	26 ± 5 (3)	0.17	0.17
Haloperidol	4.5 ± 1.62 (3)	104 (2)	0.43	0.51
Thiethylperazine	5.93 ± 2.15 (3)	73 ± 14 (3)	1.58	6.4
Loxapine	15 ± 1.75 (3)	83 (2)	ND§	3
Chlorpromazine	10 ± 1.76 (3)	151 ± 18 (3)	8.4	12
Clozapine	317 (2)	1,420 ± 173 (3)	61	24.5
Perlapine	321 (2)	3,990 ± 810 (4)	11	?
Metoclopramide	70 ± 28 (5)	16,900 (2)	8.9	12
Sulpiride	143 ± 48 (3)	29,000 (1)	176	40
U-25,927	324 (2)	1,429 ± 272 (4)	Inactive	Inactive

* Correlation between IC$_{50}$ values for [^{3}H]DHE sites and clinical doses ($r = 0.86$, $P < 0.002$); correlation between IC$_{50}$ values for [^{3}H]SPIRO sites and clinical doses ($R = 0.76$, $P < 0.015$). Correlation between IC$_{50}$ values for [^{3}H]DHE sites and ED$_{50}$ values for DOPAC elevation ($R = 0.86$, $P < 0.0015$); correlation between IC$_{50}$ values for [^{3}H]SPIRO sites and ED$_{50}$ values for DOPAC elevation ($R = 0.85$, $P < 0.002$).

† Values for IC$_{50}$ were calculated from Hill plots; (N) is the number of analyses.

‡ Values for IC$_{50}$ of [^{3}H]SPIRO sites, ED$_{50}$ for DOPAC elevation in rat striatum and clinical dose are taken from Ref. [13]. The IC$_{50}$ values for [^{3}H]SPIRO sites were calculated from Hill plots, ED$_{50}$ is the dose of drug producing a half-maximum increase in DOPAC levels in rat striatum measured at the time of peak response. [^{3}H]SPIRO was 1 to 1.5 nM; the K_d of [^{3}H]SPIRO was 0.69 nM.

§ Not determined.

macological and clinical potencies (Table 4). These sites appear to be related to the DA D2 (cyclase-independent) receptors, since the benzamide derivatives that are DA antagonists *in vivo*, but do not antagonize the stimulation of adenylate cyclase by DA, were effective in competing for [^{3}H]DHE binding.

The non-specific binding of [^{3}H]DHE, or binding in the presence of 4 nM *d*-butaclamol, was consistently nonlinear in our studies (Fig. 2). This could be due to [^{3}H]DHE binding to other saturable sites. Since [^{3}H]DHE binding reverses slowly and since a lag occurs in the association experiment, we were unable to determine the K_d of [^{3}H]DHE from kinetic experiments. Further studies are required to resolve these complexities.

The antagonism of [^{3}H]DHE binding by the benzamide derivatives is of particular interest. Previously, the benzamide derivatives metoclopramide and sulpiride were shown to be inactive in antagonizing DA stimulated adenylate cyclase [8, 18] and very weak in antagonizing [^{3}H]SPIRO binding [12, 13, 15]. As these systems are believed to represent DA receptors, the inactivity of these drugs was in contrast to their known anti-DA properties *in vivo*. For example, metoclopramide, recently shown to be an effective antipsychotic [37], increases DA turnover [8, 10, 38], stimulates prolactin release [14, 39], reverses apomorphine-induced stereotypy [40, 41], and also antagonizes the inhibitory effects of DA on vasculature [42, 43]. The antagonism of [^{3}H]DHE sites by these benzamides has, therefore, reinforced their anti-DA properties. It should be noted that with respect to its clinical dose or its ability to elevate striatal DOPAC, metoclopramide is 20 times less potent than haloperidol, but in the [^{3}H]SPIRO assay metoclopramide is 150 times weaker than haloperidol. By comparison, metoclopramide is only fifteen times weaker than haloperidol in the [^{3}H]DHE binding assay, in agreement with the studies *in vivo* (Table 4). Therefore, [^{3}H]DHE sites appear to be a good marker *in vitro* for the anti-DAergic properties of the benzamide antipsychotics. Recently, Creese *et al.* [15] reported that substituted benzamides are more potent in striatal membranes from rat and human than from calf in competing for [^{3}H]SPIRO sites. It is possible that these agents may be even more potent in competing for [^{3}H]DHE sites in rat.

In contrast to classical neuroleptics such as haloperidol and chlorpromazine, the benzamide derivatives are relatively selective DA antagonists. For example, sulpiride is a weak α-NE and 5-HT antagonist on rabbit aortic strips [44], competes poorly against cholinergic muscarinic sites labeled by [^{3}H]propylbenzylcholine and [^{3}H]QNB [28, 45], and is a weak inhibitor of [^{3}H]GABA* sites [28]. Metoclopramide and sulpiride are weak in competing for 5-HT sites labeled by [^{3}H]SPIRO in rat frontal cortex [7]. Further, metoclopramide is inactive in antagonizing the stimulation of adenylate cyclase by NE [46]. In studies on the antagonism of the effect of DA on renal vasculature, both sulpiride and metoclopramide were shown to be the most specific of the various DA antagonists evaluated [43].

The selective effect on DA receptors by these benzamides may explain their weak interaction with

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* GABA, γ-aminobutyric acid.

[^{3}H]SPIRO binding sites. Previously, we have studied binding sites labeled with [^{3}H]SPIRO as defined by 1 μM *d*-butaclamol. As spiroperidol possesses a high affinity for DA, 5-HT and α-NE sites (Table 1), the use of 1 μM *d*-butaclamol, an agent also with high affinity for those sites (Table 1), as a masking drug, would be expected to result in a mixed population of binding sites defined as "specific". We and others have found that [^{3}H]SPIRO sites are heterogeneous when 1 μM *d*-butaclamol is used to define specific binding [13, 47–49]. In such a system, a relatively non-selective drug may be expected to compete well, while a more selective drug would appear weaker. Our results are consistent with this proposition. In the present study we have found differences in the IC_{50} values of antipsychotic drugs in the [^{3}H]SPIRO and [^{3}H]DHE systems. Compared with the [^{3}H]SPIRO system, the potency of all neuroleptics has increased. This increase is approximately 10-fold for butyrophenones and phenothiazines and 5-fold for dibenzoheteroepines, whereas the increase for the more selective substituted benzamides is 200-fold (Table 3). In addition, judged by the Hill analysis, an apparent competitive inhibition was observed, in general, for antipsychotic drugs competing for DHE sites, whereas in the SPIRO system, many drugs have Hill coefficients less than one, an indication of multiple binding sites. In part, the increase of potency in the DHE assay can be attributed to the use of a lower concentration of radioligand (0.25 nM) compared to a concentration of 1 to 1.5 nM in the SPIRO assay.

The success in demonstrating an apparently homogeneous receptor population using a low *d*-butaclamol concentration and [^{3}H]DHE, a non-selective radioligand, offers an alternative method to define a subpopulation of binding sites. Previously, DA receptor binding using [^{3}H]DHE has been studied by Titeler *et al.* [30] by using phentolamine to mask α-NE sites. However, biphasic inhibition curves were found for DA and (−)NE, and the potency of DA was only two times that of (−)NE for the high affinity component of [^{3}H]DHE sites. In contrast, we found monophasic inhibition curves for DA and (−)NE with low Hill coefficients. Moreover, DA was eight times more potent than (−)NE.

In spite of the high correlation between binding and pharmacological activities for various neuroleptics (Table 4), the [^{3}H]DHE binding assay does not reflect the anti-DA properties *in vivo* of several agents. For example, *trans*-flupenthixol has no clinical efficacy [50], but is quite active in the [^{3}H]DHE assay (IC_{50} = 45 nM). U-25,927, a clinically inactive butyrophenone [51], has an IC_{50} similar to the clinically active drug clozapine. These discrepancies between *in vivo* and *in vitro* properties of antipsychotic drugs have been reported in the [^{3}H]SPIRO assay [13]. Therefore, although the DHE binding assay can better account for the anti-DA properties of substituted benzamides than can the [^{3}H]SPIRO system, it is not possible to predict the anti-DA properties of a drug based on the DHE assay alone. In this respect, it is best to combine a system using an effect *in vivo* such as elevation of striatal DOPAC levels [3] with a binding assay to reach a more valid conclusion concerning the anti-DA properties of a proposed neuroleptic agent. A similar conclusion was reached by Koe [52] in his studies on molindone.

In summary, we have demonstrated an apparent DA D2-receptor system using [^{3}H]DHE and a low concentration of *d*-butaclamol. These sites are antagonized effectively by the benzamide antipsychotics in accordance with their anti-DA potencies *in vivo*, and represent an improvement over the system labeled by [^{3}H]SPIRO as defined with a high *d*-butaclamol concentration.

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