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IRREVERSIBLE BLOCKADE OF [^{3}H]SPIPERONE- BUT NOT [^{3}H]DOPAMINE-LABELED DOPAMINE RECEPTORS WITH PHENOXYBENZAMINE

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Abstract—Calf caudate homogenates, preincubated with 10^{-5} M phenoxybenzamine followed by thorough washing, exhibited an essentially complete loss of dopamine-associated high affinity [^{3}H]spiperone specific binding. This effect of phenoxybenzamine was inhibited in a potent and concentration-dependent manner by domperidone, a potent and specific dopamine-receptor blocker. No effect of phenoxybenzamine was observed on [^{3}H]dopamine specific binding. These data indicate that the blockade of [^{3}H]spiperone binding occurred at the dopamine-receptor binding site, or sites, and that the [^{3}H]spiperone binding was molecularly distinct from the [^{3}H]dopamine specific binding. The characteristics of dopamine-associated [^{3}H]spiperone specific binding suggest that there may be two sites labeled by [^{3}H]spiperone.

In 1975–1976 the specific binding of [^{3}H]dopamine and [^{3}H]haloperidol to sites in calf brain homogenates was reported by Seeman *et al.* [1] and Burt *et al.* [2]. Burt *et al.* [2] proposed that the [^{3}H]dopamine binding site represented the agonist conformation of the brain's dopamine receptor and the [^{3}H]haloperidol specific binding site represented the antagonist conformation of the same receptor, and that these two conformations were in equilibrium with each other. Titeler *et al.* [3–5] proposed, alternatively, that the [^{3}H]dopamine specific binding site was a molecular entity distinct from the [^{3}H]haloperidol specific binding site and that the [^{3}H]haloperidol site represented the classical biological post-synaptic site of action of both agonists and antagonists of dopamine receptors. [^{3}H]Spiperone was introduced as a radioactive ligand superior to [^{3}H]haloperidol for labeling dopamine receptors in the caudate nucleus [6, 7].

Phenoxybenzamine has been shown to be a potent irreversible blocker of the α_1-adrenoreceptor [8, 9]. The following study was undertaken to investigate the effects of phenoxybenzamine on the specific dopamine-associated binding sites for [^{3}H]dopamine and [^{3}H]spiperone in calf caudate membranes in order to clarify the relationship between these two binding sites, i.e. are they molecularly distinct entities or the same receptor in different equilibrium conformations? It was also deemed of some importance to ascertain whether or not there is a moiety at, or near, either of these binding sites that is capable of an irreversible attachment with a ligand.

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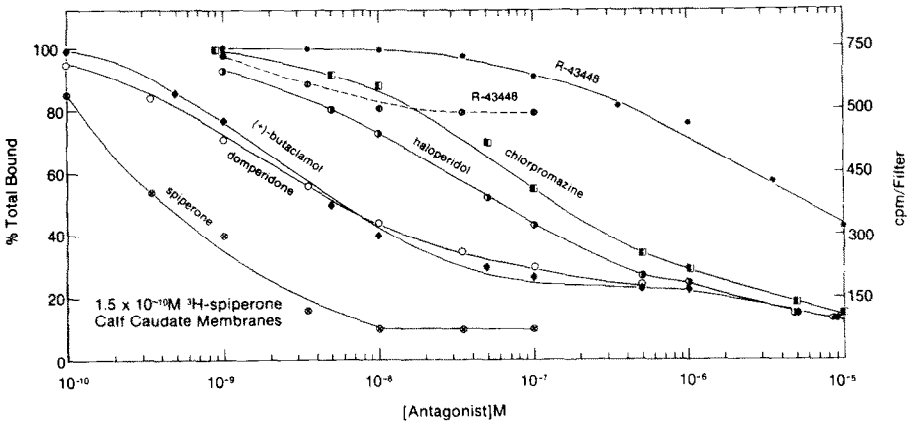
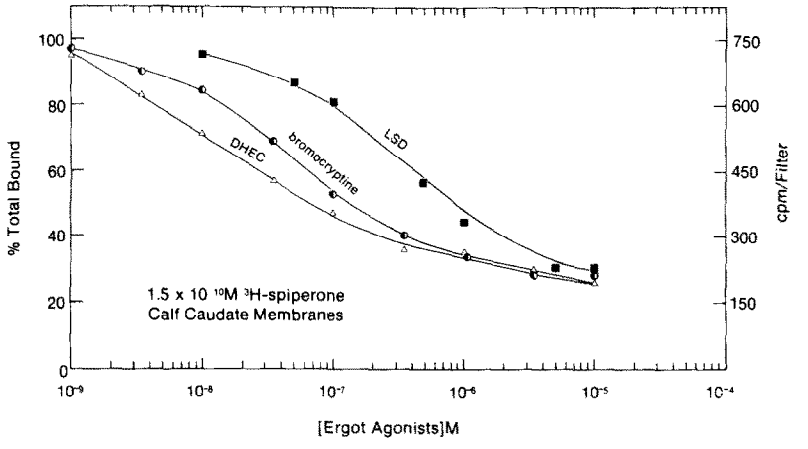
MATERIALS AND METHODS

Tissue preparation. Calf caudate crude homogenates were prepared as previously described with minor modifications [10]. Calf brains were obtained fresh from the Canada Packers Hunisett plant (Toronto, Ontario). The caudates were removed within 2 hr after death, pooled, sliced into small cubes, and suspended in buffer at an approximate concentration of 50 mg (wet weight)/ml of TEAN buffer [15 mM Tris–HCl (pH 7.4), 5 mM Na_2–EDTA, 1.1 mM ascorbate, and 12.5 μM nalamide]. The tissue was homogenized with a PT-10 polytron homogenizer, set at speed 7, for 10 sec. This homogenate was centrifuged at 39,000 *g* for 15 min and the resulting pellet was resuspended in TEAN buffer and recentrifuged twice. The final pellet was suspended in TEAN buffer to give a concentration of 100 mg wet weight/ml and was frozen for later use.

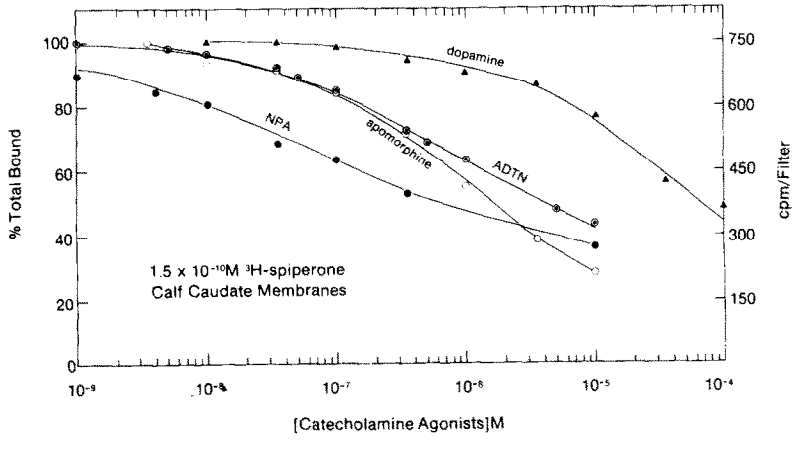
Binding assay. The specific binding of [^{3}H]spiperone and [^{3}H]dopamine was determined as previously described with minor modifications [10], by incubating [^{3}H]spiperone (New England Nuclear Corp., Boston, MA; 25 Ci/mmole), or [^{3}H]dopamine (New England Nuclear Corp.; 48 Ci/mmole), with calf caudate membranes (6 mg wet weight) in the presence or absence of competing drugs in a total volume of 0.6 ml. After a 30-min room temperature incubation, equilibrium was reached and 0.5 ml of this mixture was applied to a GF-B glass fibre filter (Whatman) under vacuum. The filters were then washed with 10 ml of TEAN buffer, immersed in A.C.S. scintillation fluid (Amersham, Arlington Heights, IL), and counted at an efficiency of 25 per cent. Specific binding was defined as the difference in cpm/filter between tubes containing radioactive drugs and membranes and tubes containing radio-



(A)



(B)



(C)

Fig. 1. Competition of drugs for 1.5×10^{-10} M [^{3}H]spiperone binding to calf caudate membranes. (A) Dopamine receptor antagonists. (B) Ergot agonists. (C) Catecholamine agonists. The dotted line in (A) indicates competition for 2×10^{-9} M [^{3}H]spiperone binding. All points were means of triplicate determinations and all S.E.M. were less than 10 per cent. The 20 per cent decline in R-43448 (A) was significant, $P < 0.01$.

active drug, non-radioactive excess drug, and membranes. For [^{3}H]spiperone the values for control membranes were approximately 770 cpm/filter, of which 75 per cent was specific. For [^{3}H]dopamine the values for control membranes were about 650 cpm/filter, of which 70 per cent was specific. All competition experiments were performed in triplicate and the S.E.M. of each point was less than 10 per cent.

Drug pretreatment and washing. The membranes were thawed on the day of the experiment, and either no drug or the preincubation drug (or drugs) was added to the membranes (30 mg wet weight/ml). They were incubated for 1 hr at room temperature. The suspension (300 mg wet weight) was diluted 100-fold with TEAN buffer and centrifuged at 39,000 *g* for 15 min. The resulting pellet was resuspended in 250 ml of buffer and recentrifuged. This procedure was performed two more times. The final pellet was suspended in 10 ml TEAN buffer, homogenized with the Polytron, and assayed for receptor binding. Protein determinations were performed using the method of Lowry *et al.* [11].

RESULTS

Figure 1 and Table 1 summarize the pharmacological characteristics of 1.5×10^{-10} M [^{3}H]spiperone binding to control calf caudate membranes. The dopamine-receptor antagonists—spiperone, haloperidol, chlorpromazine, (+)-butaclamol, and domperidone—competed for [^{3}H]spiperone-specific binding potently and produced relatively steep competition curves with Hill coefficients ranging from 0.73 to 1.06. Ergot dopamine-receptor agonists produced competition curves with properties similar to the antagonists. The catecholamine dopamine-receptor agonists—dopamine, apomorphine, ADTN* and NPA—produced relatively low potency competition curves with Hill coefficients ranging from 0.44 to 0.84. R-43448, a potent and specific 5-HT antagonist [7], did not have a potent effect on 1.5×10^{-10} M [^{3}H]spiperone specific binding, but it did inhibit 20 per cent of 2×10^{-9} M [^{3}H]spiperone specific binding. This indicates that a serotonin receptor was labeled by [^{3}H]spiperone at the higher concentrations of [^{3}H]spiperone. In Scatchard analyses of [^{3}H]spiperone binding, 5×10^{-8} M R-43448 was included to inhibit [^{3}H]spiperone binding to serotonin receptors [7, 12], as this concentration has been shown to block high affinity [^{3}H]spiperone serotonin receptors, while not interacting with dopamine receptors.

Figure 2 and Table 1 summarize the pharmacological characteristics of 10^{-9} M [^{3}H]dopamine binding to calf caudate homogenates. The catecholamines exhibited potent competition curves with Hill coefficients close to unity, as did ergot derivatives such as LSD. On the other hand, neuroleptics such as piflutixol, (+)-butaclamol, and spiperone exhibited low potency competition curves relative to their potencies in inhibiting [^{3}H]spiperone binding. There were no detectable differences in the properties of [^{3}H]spiperone or [^{3}H]dopamine binding in control membranes (non-pretreated, thoroughly washed) and membranes prepared in the standard manner (see Materials and Methods).

Figure 3A illustrates the effects of phenoxybenzamine on [^{3}H]spiperone binding in competition experiments. The IC_{50} was 1.5×10^{-7} M with a Hill coefficient of 1.5, indicating either a positive cooperativity in the interaction of phenoxybenzamine

* Abbreviations: ADTN, 2-amino-6,7-dihydroxy-(1,2,3,4)-tetrahydronapthalene; NPA, *N*-propyl-apomorphine; LSD, lysergic acid diethylamide; and DHEC, dihydroergocryptine.

Table 1. IC_{50} Values and Hill coefficients obtained from competition of drugs with 1.5×10^{-10} M [^{3}H]spiperone or 10^{-9} M [^{3}H]dopamine specific binding to calf caudate membranes*

Drug	[^{3}H]Spiperone IC_{50} (M)	[^{3}H]Spiperone Hill coefficient	[^{3}H]Dopamine IC_{50} (M)	[^{3}H]Dopamine Hill coefficient
Spiperone	4.4×10^{-10}	1.06	3.8×10^{-6}	0.56
(+)-Butaclamol	2.7×10^{-9}	0.96	1.62×10^{-7}	1.09
Haloperidol	1.7×10^{-8}	0.73		
Chlorpromazine	6.3×10^{-8}	0.77		
Promazine	1.0×10^{-7}	0.91		
Dihydroergocryptine	8.9×10^{-8}	0.81		
Bromocryptine	1.4×10^{-8}	0.81		
NPA	8.5×10^{-8}	0.44		
Apomorphine	4.0×10^{-7}	0.84	2.9×10^{-9}	1.06
ADTN	1.7×10^{-6}	0.54		
Dopamine	2.1×10^{-5}	0.74	2.4×10^{-9}	1.12
LSD	1.8×10^{-7}	1.10	2.1×10^{-8}	0.86
Piflutixol			3.6×10^{-7}	0.81
Domperidone	2.1×10^{-9}	0.80		

* [^{3}H]Spiperone specific binding was defined by 10^{-6} M (+)-butaclamol and [^{3}H]dopamine specific binding by 10^{-6} M apomorphine. Values were obtained by converting the results of three competition experiments to Hill plots by means of the Hill equation:

$$\log \frac{\% \text{ bound}}{100 - \% \text{ bound}} = \text{Hill coefficient} \times \log [\text{competing drug}].$$

All S.E.M. were less than 10 per cent.

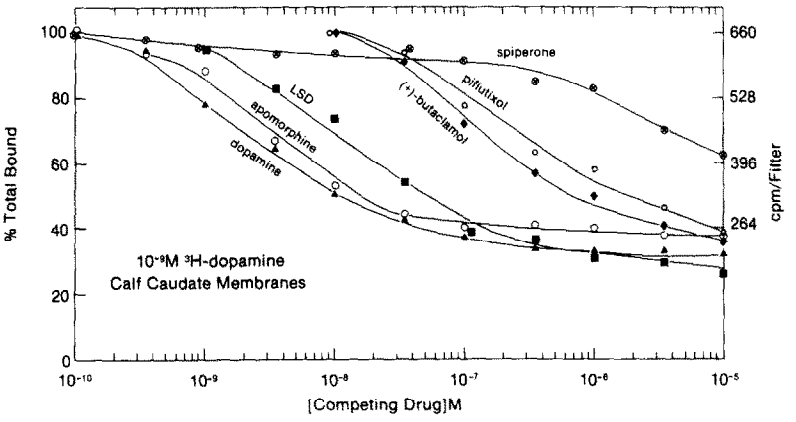


Fig. 2. Competition of drugs for 10^{-9} M [^{3}H]dopamine binding to calf caudate membranes.

with [^{3}H]spiperone binding, or an irreversible inhibition by phenoxybenzamine of [^{3}H]spiperone binding.

Figure 3B illustrates that at concentrations as high as 10^{-5} M phenoxybenzamine there was no significant effect on [^{3}H]dopamine binding in competition assays.

Table 2 presents the concentration-dependent effect of phenoxybenzamine pretreatment of calf caudate membranes on [^{3}H]spiperone binding to subsequently washed membranes. Phenoxybenzamine (10^{-5} M) was used in subsequent pharmacological experiments as this was the lowest concentration that produced near-maximal effects on [^{3}H]spiperone binding.

Figure 4 presents Scatchard analyses that demonstrate the lack of effect of 10^{-5} M phenoxybenzamine pretreatment on the saturable specific binding of [^{3}H]dopamine to subsequently washed membranes. In control and 10^{-5} M phenoxybenzamine pretreated membranes, the B_{max} and K_D were essentially the same.

Table 2. Concentration-dependent effect of phenoxybenzamine pretreatment on 1.5×10^{-10} M [^{3}H]spiperone specific binding to subsequently thoroughly washed calf caudate membranes

[Phenoxybenzamine] (M)	[^{3}H]Spiperone specific binding (fmoles/mg)	% Control
Control	54.34 ± 2	100
10^{-4}	2.87 ± 1	5.3
10^{-5}	5.14 ± 2	9.5
10^{-6}	37.53 ± 2	69
10^{-7}	49.85 ± 3	92

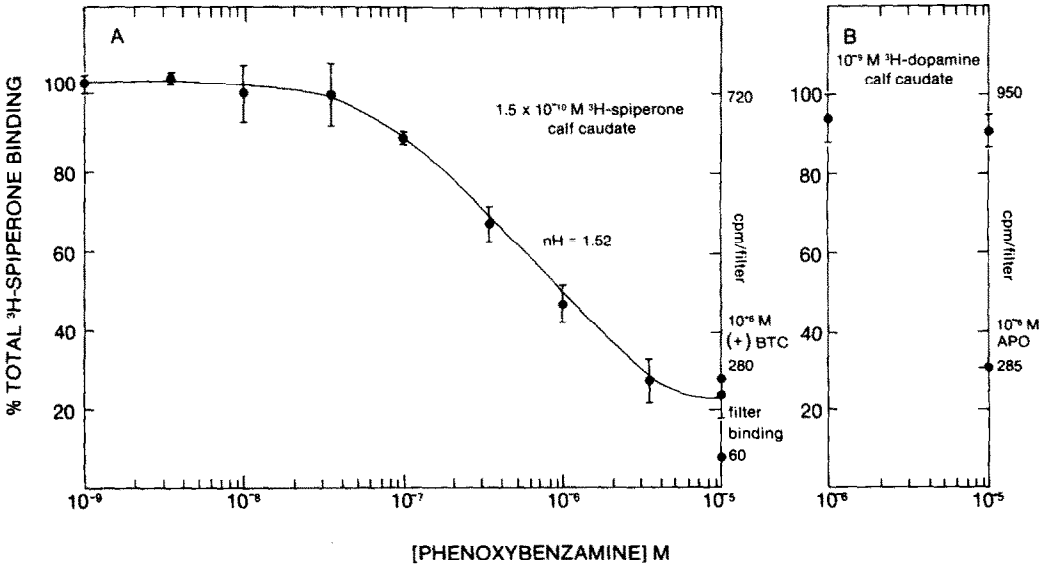


Fig. 3. (A) Competition by phenoxybenzamine for 1.5×10^{-10} M [^{3}H]spiperone binding to calf caudate membranes. (B) Competition by phenoxybenzamine for 10^{-9} M [^{3}H]dopamine binding to calf caudate membranes.

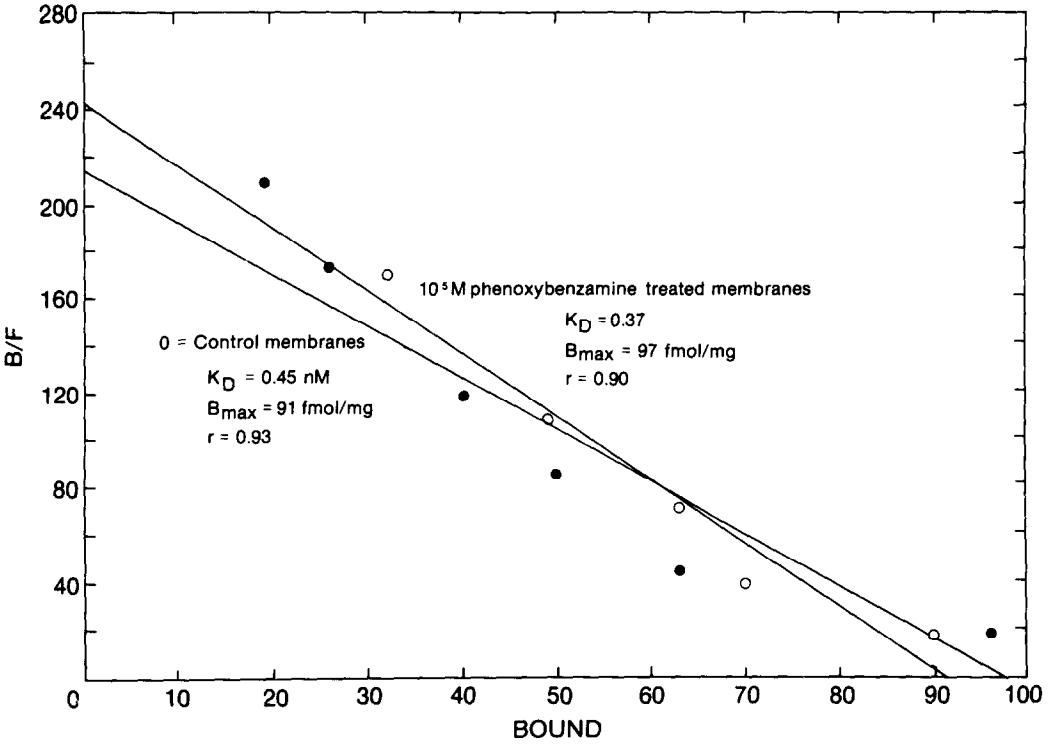


Fig. 4. Scatchard analysis of [^{3}H]dopamine specific binding (defined by 10^{-6} M apomorphine) to calf caudate membranes pretreated and non-pretreated with 10^{-5} M phenoxybenzamine and then thoroughly washed. The results are from triplicate determinations, and all S.E.M. were less than 15 per cent.

Figure 5 and Table 3 present Scatchard analyses that illustrate the effect of 10^{-5} M phenoxybenzamine pretreatment on saturable [^{3}H]spiperone binding (in the presence of 5×10^{-8} M R-43448). Control membranes (line A) exhibited a B_{max} of 384 fmoles/mg, with a K_D of 4×10^{-10} M, while membranes pretreated with 10^{-5} M phenoxybenzamine (line F) exhibited a B_{max} of 97 fmoles/mg, with a K_D of 1.27×10^{-9} M. The inclusion of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M domperidone (lines B through E respectively) with phenoxybenzamine in the pretreatment step resulted in a reversal of the inhibition of [^{3}H]spiperone binding to the subsequently washed membranes. Not shown is the effect of 10^{-6} M domperidone in the absence of phenoxybenzamine pretreatment on subsequently washed membranes. There was no detectable difference between these membranes and non-pretreated membranes. A small amount of specific binding (97 fmoles/mg) was left after 10^{-5} M phenoxybenzamine treatment, and this binding displayed an apparent lower affinity (K_D = 1.27×10^{-9} M) than control membranes. The pharmacology of this binding has not yet been investigated.

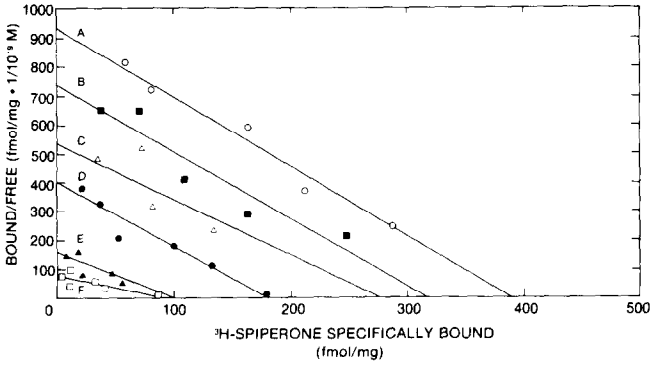


Fig. 5. Reversal of phenoarylbenzamine blockade by domperidone. Scatchard analysis of [^{3}H]spiperone specific binding (defined by 10^{-6} M (+)-butaclamol) to thoroughly washed calf caudate membranes. Key: (A) non-pretreated; (B) 10^{-5} M phenoxybenzamine + 10^{-6} M domperidone-pretreated; (C) 10^{-5} M phenoxybenzamine + 10^{-7} M domperidone-pretreated; (D) 10^{-6} M phenoxybenzamine + 10^{-8} M domperidone-pretreated; (E) 10^{-5} M phenoxybenzamine + 10^{-9} M domperidone-pretreated; (F) 10^{-5} M phenoxybenzamine-pretreated. The results were from triplicate determinations, and all S.E.M. were less than 20 per cent.

Table 3. B_{max} and K_D values obtained for [^{3}H]spiperone specific binding to calf caudate membranes after pretreatment and thorough washing of the membranes*

Pretreatment	Bound (fmoles/mg)	K_D (M)
None	384 ± 15	4.1×10^{-10}
10^{-5} M Phenoxybenzamine	97 ± 4	1.27×10^{-9}
10^{-5} M Phenoxybenzamine + 10^{-9} M domperidone	86 ± 6	5.4×10^{-10}
10^{-5} M Phenoxybenzamine + 10^{-8} M domperidone	179 ± 23	4.5×10^{-10}
10^{-5} M Phenoxybenzamine + 10^{-7} M domperidone	280 ± 22	5.2×10^{-10}
10^{-5} M Phenoxybenzamine + 10^{-6} M domperidone	318 ± 36	4.3 ± 10^{-10}

* [^{3}H]Spiperone binding was performed in the presence of 5×10^{-8} M R-43448 to prevent [^{3}H]spiperone binding to the 5-HT receptor. Pretreatment with domperidone alone had no significant effect on the binding relative to non-pretreated tissues (data not shown). Results were from triplicate determinations. All S.E.M. were less than 20 per cent.

DISCUSSION

Preliminary results of the effects of phenoxybenzamine on dopamine receptors have been reported previously [13–16]. Marchais and Bockaert [13] demonstrated that the dopamine-sensitive cyclase could be stimulated although all [^{3}H]spiperone specific binding has been irreversibly blocked by phenoxybenzamine, indicating that the [^{3}H]spiperone site is not the recognition site of the dopamine-sensitive cyclase. Hamblin and Creese [14] demonstrated that 10^{-5} M phenoxybenzamine could irreversibly block all [^{3}H]spiperone binding, partially block [^{3}H]apomorphine specific binding and not block [^{3}H]dopamine binding, indicating that possibly part of the [^{3}H]apomorphine binding is to the [^{3}H]spiperone sites and that the [^{3}H]dopamine binding is to another site. Titeler [15] demonstrated irreversible inhibition of 1.5×10^{-10} M [^{3}H]spiperone specific binding by 10^{-5} M phenoxybenzamine with no apparent effects of 10^{-5} M phenoxybenzamine on [^{3}H]dopamine specific binding. Spiperone (10^{-7} M) was shown to reverse the effect of 10^{-5} M phenoxybenzamine.

In this study, 10^{-5} M phenoxybenzamine clearly irreversibly eliminated the saturable specific binding of [^{3}H]spiperone to dopaminergic sites in calf caudates. The concentration-dependent reversal of the effect by domperidone, a specific dopamine receptor antagonist [17] indicates that phenoxybenzamine was irreversibly attached at, or near, the [^{3}H]spiperone binding site, and that the phenoxybenzamine effect therefore included a specific effect at the receptor binding site. This is an important point, as phenoxybenzamine is an extremely nonspecific alkylating agent [18]. Since [^{3}H]dopamine specific binding was unaffected by phenoxybenzamine, it is quite clear that the [^{3}H]dopamine binding site was distinct from the [^{3}H]spiperone binding site. This conclusion is supported by other evidence in the literature, including the following: (1) the [^{3}H]spiperone binding site is detected in the pituitary but the [^{3}H]dopamine binding site is not [19]; (2) thermodynamic arguments argue strongly against the [^{3}H]dopamine and [^{3}H]spiperone sites being in any equilibrium [20]; and (3) centrifugation studies result in differential enrichments of the two sites in different fractions [21].

The irreversible interaction of phenoxybenzamine with the specific [^{3}H]spiperone site indicates that there was a functional moiety at the binding site capable of irreversible interaction with an alkylating agent. This indicates that irreversible ligands for the dopamine receptor of a more specific nature may be developed in the future.

Several important pharmacological properties of the specific [^{3}H]dopamine binding detected in this study should be noted. This binding site displays no high affinity component for the potent dopamine-sensitive cyclase antagonist piflutixol [22]. This indicates that the specific [^{3}H]dopamine binding was apparently not to the dopamine-sensitive adenylate cyclase. The low affinity that piflutixol exhibited ($IC_{50} = 3.6 \times 10^{-7}$ M) is much lower than that reported by Burt *et al.* [2] ($K_i = 6.7 \times 10^{-8}$ M). Moreover, the affinity of [^{3}H]dopamine for the site detected in this study ($K_D = 4 \times 10^{-10}$ M) and the B_{max} (91 fmoles/mg; 3 pmoles/g wet weight) are significantly different from those reported by Burt *et al.* [2] ($K_D = 1.7 \times 10^{-8}$ M, $B_{max} = 13$ pmoles/g wet weight). It is thus quite clear that the methodology used in this study resulted in [^{3}H]dopamine labeling a site or sites with properties different from the site or sites that have been detected using the methodology of Burt *et al.* [2]. The importance of tissue preparation and assay conditions in binding studies utilizing radioactive catecholamines has been emphasized in a previous publication [10]. Spiperone, a potent D_2 dopamine-receptor antagonist, was shown to have little or no effect at low concentrations (Fig. 2). This indicates that the majority of the [^{3}H]dopamine specific binding under these conditions was not to the dopamine receptor labeled by [^{3}H]spiperone.

The [^{3}H]spiperone binding presented here revealed complex binding characteristics. Neuroleptics and ergots generally produce steeper competition curves than catecholamines. Yet the Hill coefficients of haloperidol and chlorpromazine were 0.73 and 0.77, respectively, indicating that, while their competition curves were steeper than catecholamine competition curves, they were in fact somewhat shallower than predicted for the competition for one binding site for [^{3}H]spiperone. It is possible, therefore, that there are actually two high affinity dopamine-associated [^{3}H]spiperone binding sites

labeled. These sites have similar affinities for neuroleptics and ergots, but different affinities for catecholamines. This hypothesis has been formulated for [^{3}H]haloperidol and [^{3}H]spiperone binding by Titeler *et al.* [23]. Recently Sibley and Creese [24] have provided further evidence for multiple dopamine-associated [^{3}H]spiperone binding sites in pituitary tissue.

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