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Phenoxybenzamine Treatment Differentiates Dopaminergic ^{3}H-Ligand Binding Sites in Bovine Caudate Membranes

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SUMMARY

Phenoxybenzamine, the classic *alpha*-adrenergic receptor alkylating agent, also acts as an irreversible antagonist of the binding of [^{3}H]spiroperidol, a D-2-selective dopaminergic ligand, to bovine caudate membranes. Doses completely eliminating the binding of this ligand leave the binding of [^{3}H]dopamine to D-3 sites virtually unaffected. The binding sites for these two ligands thus represent distinct subtypes of dopamine receptors, not interconverting states of a single receptor. This phenoxybenzamine-mediated inhibition proceeds via a dose-dependent (pseudo-IC_{50} = 1 μM) decrease in B_{max} with little or no change in affinity for ^{3}H-ligands at the D-2 site. The effect is site-directed, as the dopaminergic agonists dopamine and apomorphine and the antagonist domperidone are able to protect against phenoxybenzamine-mediated attack in proportion to their affinities for D-2 sites. Epinephrine, norepinephrine, and serotonin are much less effective in protecting these sites. The sensitivity of [^{3}H]apomorphine binding is intermediate to that of [^{3}H]spiroperidol and [^{3}H]dopamine. [^{3}H]Apomorphine binding can be resolved into a phenoxybenzamine-labile population of binding sites which have equal phenoxybenzamine sensitivity, selectivity among protecting agents, and butyrophenone affinity to those of D-2 sites labeled by ^{3}H-butyrophenones, and a separate phenoxybenzamine-stable population of sites which have an affinity for dopamine comparable to that of D-3 sites labeled by [^{3}H]dopamine. [^{3}H]Apomorphine therefore appears to label a portion of D-2 receptor sites in addition to D-3 receptors.

INTRODUCTION

Several lines of evidence, including radioligand binding techniques, suggest the existence of multiple dopamine receptor subtypes (1). One such putative subtype found in caudate and other brain regions possesses high affinity for [^{3}H]dopamine and other dopaminergic agonists but low affinity for butyrophenone antagonists (2, 3) and has recently been termed the D-3 binding site (3). D-3 sites may thus be labeled in filtration binding assays by [^{3}H] dopamine and other agonist radioligands but not by ^{3}H-butyrophenones. The ^{3}H-butyrophenone ligands instead appear to label a second class of binding sites, present in both pituitary and brain, called D-2 binding sites (2, 3). For these sites, the rank order of drug affinities is reversed: D-2 sites are labeled with high affinity by [^{3}H] spiroperidol and other ^{3}H-butyrophenones, while having insufficient affinity for dopamine to be practically labeled by [^{3}H]dopamine itself. An adenylate cyclase-linked, or D-1, receptor subtype appears to be a distinct entity not labeled by either ^{3}H-butyrophenones or ^{3}H-agonists (1, 4). Important questions in the interpretation of these studies have been whether D-2 and D-3 dopaminergic binding sites represent distinct molecular entities or interconverting states of the same receptor (5), and whether the various ^{3}H-agonist ligands in use label the same or different populations of sites.

Recently, we described in a preliminary report (6) a method to inactivate selectively and apparently irreversibly subsets of dopaminergic binding sites using phenoxybenzamine, suggesting a definitive approach to these questions. Phenoxybenzamine, a classic irreversible *alpha*-adrenergic antagonist, was noted as long ago as 1967 to block the dopamine-mediated inhibition of caudate neuron cell firing (7). With the advent of the neurotransmitter receptor binding technique, the drug was found to inhibit potently ligand binding to dopaminergic binding sites (8) and shortly thereafter to prevent stimulation of the dopamine-linked adenylate cyclase (9).

We now detail the effects of phenoxybenzamine treatment on dopaminergic ^{3}H-ligand binding to D-2 and D-3 binding sites in bovine caudate homogenates. Data are presented confirming evidence from other binding studies that the D-2 and D-3 sites labeled by ^{3}H-butyrophenones and [^{3}H]dopamine, respectively, are physically distinct

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and do not interconvert under the conditions of the assay. In addition, the agonist [^{3}H]apomorphine is shown to label a portion of the D-2 sites of caudate membranes, as has previously been demonstrated for anterior pituitary membranes (10).

METHODS

Tissue preparation. Bovine caudate was dissected from fresh brains at the slaughterhouse and frozen at −70° within 4 hr of death. The frozen tissue was used between 1 and 60 days after freezing, during which time binding parameters remained unchanged. On the day of assay the tissue was homogenized in 50 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.6 at 25°) at 4° with a Tekmar homogenizer (setting 7 for 10 sec) and centrifuged twice (10 min at 50,000 × *g*, in a Sorvall RC-5B refrigerated centrifuge) with intervening resuspension in fresh buffer. Except where stated otherwise, throughout the procedure all drugs and tissue preparations were constantly maintained at 0–4°.

Reaction of homogenates with phenoxybenzamine. For reaction with phenoxybenzamine the pellet was then suspended in fresh Tris buffer containing 0.1% ascorbic acid, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (assay buffer) at a concentration of 37.5 mg of tissue (wet weight) per milliliter. Aliquots containing protecting drugs, with 10 μM pargyline, for protection experiments were preincubated for 10 min at 37°, and phenoxybenzamine [dissolved less than 10 min prior to use in distilled water containing 0.1% ascorbic acid and 10% ethanol (v/v)] was then added. Homogenate ethanol concentration did not exceed 0.25% (v/v), a concentration more than 10 times lower than that required to alter detectably the binding characteristics of any ^{3}H-ligand studied. Appropriate carrier solution was added to controls. The reaction was terminated after 10 minutes (or other periods for time course experiments) with the addition of 10 volumes of 0° assay buffer followed immediately by centrifugation as before. Tissue homogenates were then washed one additional time in fresh assay buffer by suspension and recentrifugation for all studies except those in which other drugs were present (protection experiments), when five washes were performed to remove such drugs.

Radioligand binding. Binding methods were similar to those described previously (8, 11, 12). All assays were performed after washing to remove phenoxybenzamine, as described above. The pellet resulting from the final centrifugation after the treatments was resuspended in assay buffer (including 10 μM pargyline for [^{3}H]dopamine binding) at 6–12 mg of tissue (wet weight) per milliliter, a concentration determined to be within the range of tissue linearity with respect to specific and nonspecific binding. All ^{3}H-ligands and competing drugs were made up just prior to use in fresh 0.1% ascorbic acid in distilled water. Binding was initiated by addition of 800 μl of tissue suspension to 200 μl of ^{3}H-ligand solution with or without competing drugs, immediately followed by thorough mixing and incubation in a 37° water bath for 15 min. This incubation period was sufficient for the association and dissociation of all ligands to reach equilibrium. Incubation was terminated by rapid filtration under reduced pressure through 2.4-cm Whatman GF/B filters followed by three 5-ml rinses of ice-cold 50 mM Tris-HCl buffer, pH 7.6 at 25°. Filters were extracted with agitation for 2 hr. in Beckman Bantam vials containing 4 ml of Beckman Ready-Solv and then counted at efficiencies of 46–50% in a Beckman LS7500 liquid scintillation spectrometer.

Nonspecific binding was determined as that binding not displaceable by (+)-butaclamol at 10^{-6} M for [^{3}H]spiroperidol, [^{3}H]haloperidol, [^{3}H]domperidone, and [^{3}H]flupentixol, and 10^{-5} M for [^{3}H]apomorphine and [^{3}H]dopamine. These respective concentrations of (+)-butaclamol were determined to be appropriate by displacement experiments, including comparisons with (−)-butaclamol and other dopaminergic drugs as reported in part elsewhere (8, 11, 12) and performed separately for the system used in these studies. At the 0.5 nM [^{3}H]spiroperidol concentration used in all studies other than saturation experiments, displacement studies revealed that binding was predominantly dopaminergic, with 15% or less of (+)-butaclamol-displaceable binding to serotonergic (5-HT_2[3]) sites. For [^{3}H]spiroperidol saturations, which required higher ligand concentrations, 10 μM ADTN was used as the blank, to displace selectively dopaminergic, but not serotonergic, binding (13). That ADTN was an appropriate blank was demonstrated by the nearly identical results obtained using another method to detect selectively dopaminergic [^{3}H]spiroperidol binding. R41468 (30 nM), a serotonergic antagonist useful for its high selectivity for 5-HT_2 receptors (14), could also be included in the assay to eliminate the confounding serotonergic binding, with (+)-butaclamol used as the blank. In our hands, this compound displaces [^{3}H]spiroperidol with a plateau (around 30 nM) at the same level as plateaus in the displacement curves of two other serotonergic antagonists, mianserin and cinanserin. This high-affinity serotonergic displacement by R41468 is not additive with that of the other two serotonergic compounds, but it is additive with the dopaminergic displacement by 10 μM ADTN.[4] Thus this concentration of R41468 selectively inhibited serotonergic [^{3}H]spiroperidol binding. It furthermore had no detectable effect on the binding of any other dopaminergic ^{3}H-ligand used. All determinations of both specific and nonspecific binding were performed in triplicate, with standard errors of the mean typically less than 3%. Specific binding to control membranes accounted for approximately 55–65% (800 cpm) for [^{3}H]domperidone, 72–90% (300–1800 cpm) for [^{3}H]spiroperidol, 40–50% (1100 cpm) for [^{3}H]haloperidol, 40% (3000 cpm) for [^{3}H]flupentixol, 40–70% (450–3500 cpm) for [^{3}H]apomorphine, or 45–65% (1600 cpm) for [^{3}H]dopamine of control total binding, with the lower figure referring to that for the highest concentration of ligand used in saturation studies, and the higher figure referring to that for the lower concentration of ^{3}H-ligand used in all other experiments. Unless stated otherwise, results are given as means ± standard error of the mean. Statistical significance was determined using a two-tailed Student's *t*-test.

[3] The abbreviations used are: 5-HT_2, serotonin; ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene.

[4] M. W. Hamblin, S. E. Leff, and I. Creese, manuscript in preparation.

The ligands used were [^{3}H]domperidone (59.9 Ci/mmole), [^{3}H]spiroperidol (25.5 or 39.0 Ci/mmole), [^{3}H]haloperidol (7.9 Ci/mmole), [^{3}H]apomorphine (28.7 or 30.0 Ci/mmole), and [^{3}H]dopamine (44.0 or 48.0 Ci/mmole) and were purchased or were gifts from New England Nuclear Corporation (Boston, Mass.); [^{3}H]flupentixol (17.2 Ci/mmole), from the Nuclear Research Centre, Negev, Israel, was kindly provided by Dr. J. Hyttel.

Materials. The sources of the drugs were as follows: apomorphine, gift of Merck and Company (Rahway, N. J.); (+)- and (−)-butaclamol, gift of Ayerst Laboratories (New York, N. Y.); domperidone and R41468, gift of Janssen Pharmaceutica (Beerse, Belgium); dopamine hydrochloride, epinephrine bitartrate, 5-hydroxytryptamine-creatinine sulfate complex, norepinephrine bitartrate, and pargyline hydrochloride, Sigma Chemical Company (St. Louis, Mo.); dibenamine and phenoxybenzamine hydrochloride, gifts of Smith, Kline & French Laboratories (Philadelphia, Pa.).

RESULTS

Time course of phenoxybenzamine inhibition of [^{3}H]spiroperidol, [^{3}H]dopamine, and [^{3}H]apomorphine binding. Incubation of homogenates with 3 μM phenoxybenzamine rapidly reduced subsequent specific binding of the butyrophenone antagonist [^{3}H]spiroperidol and the agonist [^{3}H]apomorphine, but not that of [^{3}H]dopamine (Fig. 1). The reduction in [^{3}H]apomorphine binding was complete after 1 min of exposure, the earliest time point examined, whereas the reduction in [^{3}H]spiroperidol binding was essentially complete after about 10 min. Incubation with phenoxybenzamine for more than 10 min produced no additional change in ligand binding regardless of drug concentration employed, so this incubation period was used in all further studies. The inactivation of [^{3}H]spiroperidol binding appeared to proceed with first-order kinetics and to have a half-time of 3 min at 3 μM phenoxybenzamine.

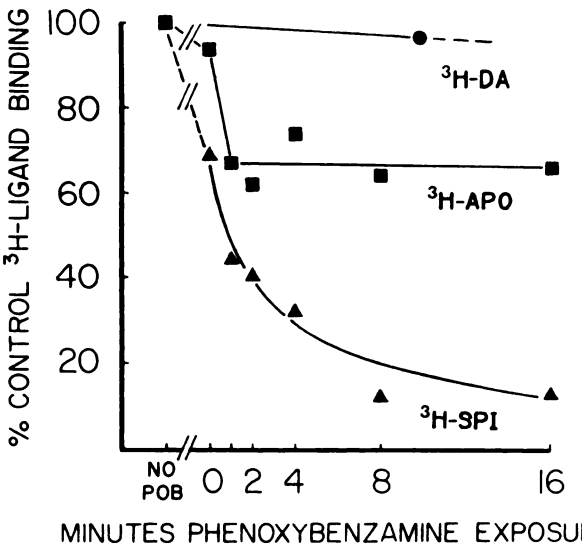

FIG. 1. *Time course for phenoxybenzamine (POB) inhibition of [^{3}H]spiroperidol (^{3}H-SPI), [^{3}H]dopamine (^{3}H-DA), and [^{3}H]apomorphine (^{3}H-APO) binding*

All tubes were incubated at 37° for a total of 42 min. Control solution or 3 μM phenoxybenzamine was added at various times prior to dilution and cooling as described under Methods. The *0 min* value represents an equivalent amount of phenoxybenzamine added to a tube immediately after dilution and cooling. Membranes were then thoroughly washed before assay for binding. Results are expressed as percentages of specific binding of [^{3}H]spiroperidol (0.5 nM), [^{3}H]dopamine (3.0 nM), and [^{3}H]apomorphine (0.8 nM) to homogenates treated in parallel with addition of control solution. Points shown are means of two separate experiments with standard errors of the mean less than 10%.

Dose response of phenoxybenzamine inhibition of [^{3}H]spiroperidol, [^{3}H]domperidone, [^{3}H]haloperidol, and [^{3}H]flupentixol. Phenoxybenzamine pretreatment for 10 min caused similar decreases in binding of the D-2-selective ^{3}H-ligands [^{3}H]spiroperidol, [^{3}H]domperidone, and [^{3}H]haloperidol and the putatively D-1-selective ligand [^{3}H]flupentixol, with pseudo-IC_{50} values ranging from 0.45 μM to 0.98 μM (data not shown). Note that these figures are higher than the K_i of phenoxybenzamine at *alpha*$_1$-receptors (3 nM) and at *alpha*$_2$-receptors (31 nM) (15). Two small but significant differences in the susceptibility of the binding of the dopaminergic antagonist ligands to phenoxybenzamine elimination were noted. First, phenoxybenzamine displayed a pseudo-IC_{50} for the inhibition of [^{3}H]domperidone binding, a ligand thought to be completely D-2-specific (16), significantly lower than for that of [^{3}H]spiroperidol, a ligand labeling not only D-2 sites, but, to a lesser extent, striatal serotonergic sites as well (17, 18). Second, after treatment with 10 μM phenoxybenzamine, which left only 5.3 ± 0.4% (n = 3) of [^{3}H]spiroperidol specific binding intact and eliminated [^{3}H]domperidone specific binding completely, a small but significant portion, 19 ± 4% (n = 4), of [^{3}H]flupentixol specific binding remained.

This inhibition of dopaminergic antagonist binding is relatively specific to phenoxybenzamine, since dibenamine, another closely related *alpha*-adrenergic alkylating agent, was approximately 50 times less potent than phenoxybenzamine in reducing [^{3}H]spiroperidol binding. The magnitude of phenoxybenzamine inhibition was also inversely related to tissue concentration, with 1 μM phenoxybenzamine causing > 80% inhibtion of [^{3}H]spiroperidol binding in homogenates at 1 mg of tissue per milliliter rather than the approximately 50% inhibition seen in the above experiments at 37.5 mg of tissue per milliliter.

Dose response of phenoxybenzamine inhibition of ^{3}H-agonist binding. The potency of phenoxybenzamine in eliminating the specific binding of two dopaminergic ^{3}H-agonists differed greatly, as it did in its time course, but was less for either agonist than for the inhibition of [^{3}H]spiroperidol binding (Fig. 2A). Phenoxybenzamine almost completely discriminated between [^{3}H]spiroperidol and [^{3}H]dopamine binding at a pretreatment phenoxybenzamine concentration of 10 μM. Such pretreatment of homogenates for 10 min eliminated nearly all specific binding of [^{3}H]spiroperidol, while reducing specific binding of [^{3}H]dopamine by only 13%. [^{3}H]Apomorphine binding showed sensitivity intermediate to that of [^{3}H]dopamine and [^{3}H]spiroperidol binding. This suggested that [^{3}H]apomorphine was labeling both the phenoxybenzamine-resistant sites labeled by [^{3}H]dopamine (D-3) and the relatively phenoxybenzamine-sensitive sites labeled by [^{3}H]spiroperidol (D-2). Were this the case, it

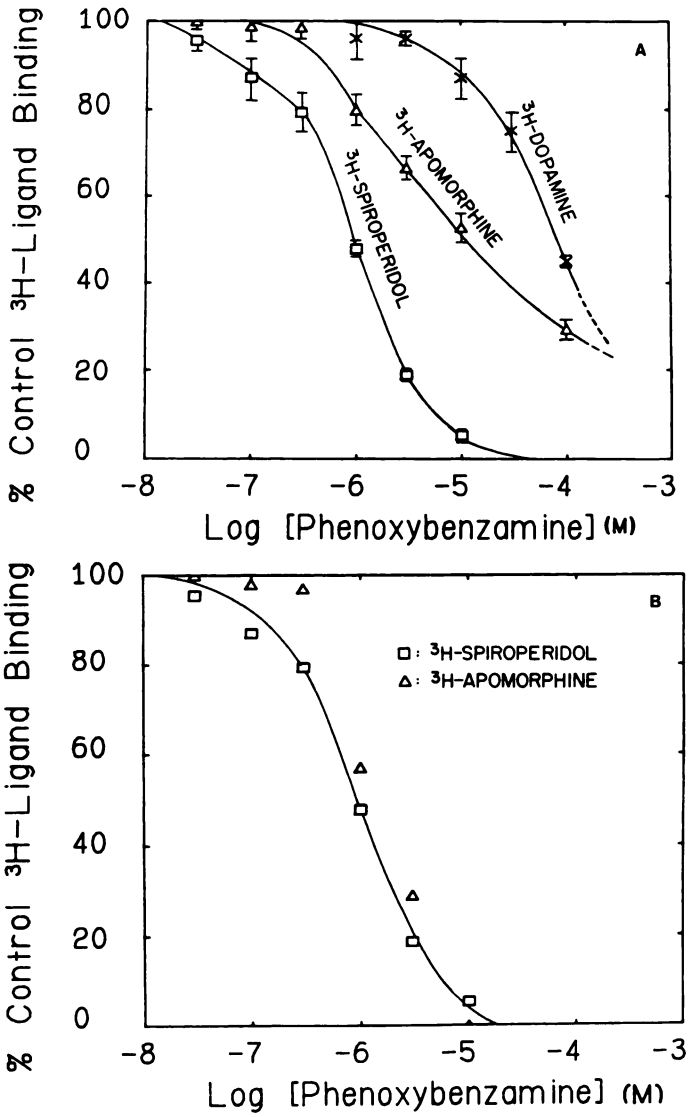

FIG. 2. *Phenoxybenzamine inhibition of [³H]spiroperidol, [³H] apomorphine, and [³H]dopamine specific bindings*

Phenoxybenzamine was most potent in eliminating specific binding of antagonists such as [³H]spiroperidol and least potent in decreasing that of [³H]dopamine. Potency at [³H]apomorphine binding sites was intermediate to that for [³H]spiroperidol and [³H]dopamine sites.

A. Results are expressed as percentages of control specific ³H-ligand binding remaining after exposure of homogenates to various concentrations of phenoxybenzamine for 10 min followed by thorough washing. Concentrations of ligands used were 0.5 nM [³H]spiroperidol, 0.8 nM [³H]apomorphine, and 3 nM [³H]dopamine. Each point represents the mean ± standard error of the mean of three to five independent determinations.

B. Data are the same as above, with phenoxybenzamine-labile binding defined as that [³H]apomorphine-specific binding inhibited by treatment with 10 μM phenoxybenzamine. Phenoxybenzamine-labile binding is thus derived from data in A as (% [³H]apomorphine specific binding remaining in treated membranes − binding to 10 μM phenoxybenzamine-treated membranes)/(control specific binding − binding to 10 μM phenoxybenzamine-treated membranes) × 100. The [³H]spiroperidol curve is identical with that shown in A and is repeated for comparison. Results are expressed as means of four or five independent determinations with standard error of the mean less than 8%.

would be necessary, but not sufficient, to show that the D-2-specific portion of the [³H]apomorphine binding possessed sensitivity to phenoxybenzamine similar to D-2 sites as measured using [³H]spiroperidol binding. Using the relatively D-2-selective 10 μM phenoxybenzamine treatment to define "phenoxybenzamine-labile" [³H]apomorphine specific binding (about 53% of the total) which could be to D-2 sites, the phenoxybenzamine dose-response curve of the phenoxybenzamine-labile [³H]apomorphine binding sites followed closely that for [³H] spiroperidol binding sites (Fig. 2B). Thus, although there was no obvious plateau in the [³H]apomorphine-phenoxybenzamine sensitivity curve, these results were at least consistent with the suggestion that [³H]apomorphine labeled phenoxybenzamine-sensitive D-2 sites, as well as relatively phenoxybenzamine-resistant D-3 sites.

Saturation studies with [³H]spiroperidol and [³H] apomorphine after homogenate treatment with phenoxybenzamine. Treatment with phenoxybenzamine reduced the B_{max} of binding for both [³H]spiroperidol and [³H] apomorphine in a dose-dependent fashion (Fig. 3A and B). At the 10 μM concentration, which eliminated virtually all 9 pmoles/g of [³H]spiroperidol-labeled D-2 sites, only about 7 pmoles/g of [³H]apomorphine sites were eliminated. This latter value thus represents an upper limit to the possible density of [³H]apomorphine-labeled D-2 sites, and indicates that, if [³H]apomorphine labels D-2 receptors in caudate, it labels only a portion of them. The total number of binding sites (B_{max}) in control membranes was 15.5 ± 1.0 pmoles/g ($n = 4$) for [³H]apomorphine and 9.0 ± 0.3 pmoles/g ($n = 2$) for [³H]spiroperidol. There was no significant change in K_D for the residual binding of [³H]apomorphine, with respective affinities for control and 1 μM phenoxybenzamine-treated homogenates of 4.44 ± 0.13 ($n = 4$) nM and 4.67 ± 0.11 ($n = 2$) nM. We previously reported (6) that phenoxybenzamine treatment left the affinity of residual [³H]spiroperidol binding sites unchanged, when binding was defined with 1 μM (+)-butaclamol as blank. As [³H]spiroperidol is also known to bind to serotonergic 5-HT_2 receptors, we have recently repeated these experiments under binding conditions selective for dopaminergic sites. Using 10 μM ADTN as blank, phenoxybenzamine again lowered the [³H]spiroperidol B_{max}, but caused a slight increase in K_D from 0.080 ± 0.007 nM for control homogenates to 0.149 ± 0.012 nM for 1 μM phenoxybenzamine-treated homogenates (significantly different, $p < 0.025$) (Fig. 3B).

Displacement of [³H]apomorphine by spiroperidol and domperidone after homogenate exposure to phenoxybenzamine: definition of high-affinity D-2 and D-3 [³H]apomorphine binding. That phenoxybenzamine-labile [³H]apomorphine binding sites displayed a phenoxybenzamine sensitivity similar to that of [³H]spiroperidol binding suggested that [³H]apomorphine labeled in part the D-2 sites. If so, it would be anticipated that the affinity of spiroperidol in displacing phenoxybenzamine-labile [³H]apomorphine binding would be identical with the affinity of [³H]spiroperidol for butyrophenone binding sites determined directly by saturation studies. As reported elsewhere (19, 20), displacement of [³H]apomorphine-specific binding from control membranes by butyrophenones was biphasic with an over-all pseudo-

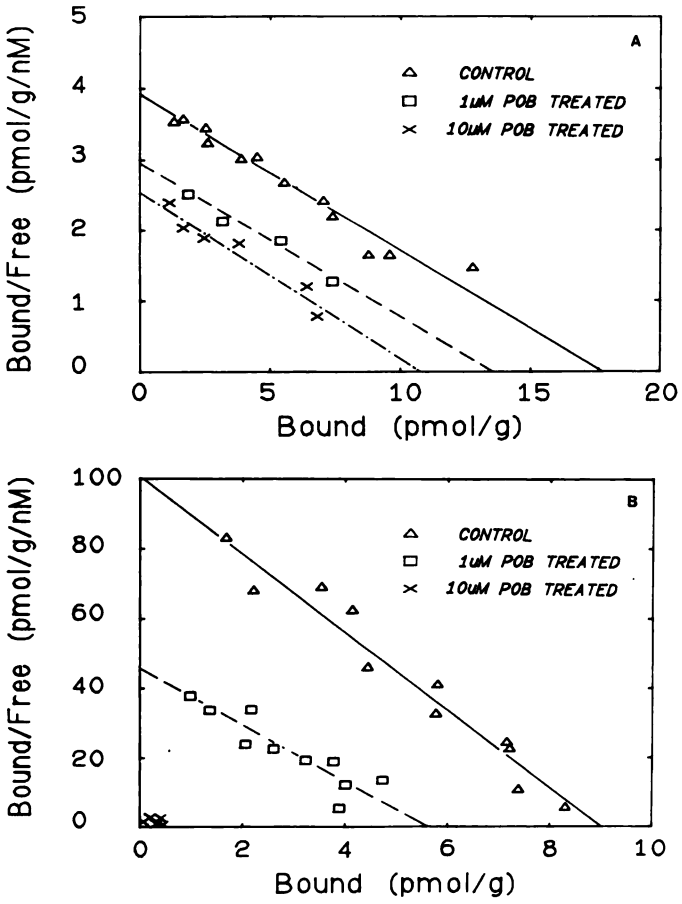

FIG. 3. *Saturation of [^{3}H]apomorphine (A) and [^{3}H]spiroperidol (B) binding in control and phenoxybenzamine (POB)-treated homogenates*

Homogenates were preincubated for 10 min with various concentrations of phenoxybenzamine or vehicle solution and then washed thoroughly before assay for binding as described under Methods. The concentrations of ligands used were 0.04–1.6 nM for [^{3}H]spiroperidol and 0.5–10 nM for [^{3}H]apomorphine. Results are presented as Scatchard plots of typical experiments. Specific binding was that displaceable by 10 μM (±)-ADTN for [^{3}H]spiroperidol and 10 μM (+)-butaclamol for [^{3}H]apomorphine.

Hill slope for spiroperidol of about 0.5, suggesting the presence of more than one type of [^{3}H]apomorphine binding site (Fig. 4A). Treatment with 10 μM phenoxybenzamine for 10 min, which eliminated 95% of all [^{3}H] spiroperidol high-affinity binding, eliminated only that [^{3}H]apomorphine binding displaceable with high affinity by unlabeled spiroperidol (Fig. 4A). Such treatment had no significant effect on those [^{3}H]apomorphine sites with low affinity for spiroperidol. Approximately 40–50% of the total [^{3}H]apomorphine-specific binding sites were thus distinguishable from the remainder by two characteristics—high affinity for spiroperidol and susceptibility to phenoxybenzamine inhibition. Log-probit plots of spiroperidol displacement of [^{3}H]apomorphine from the phenoxybenzamine-labile sites of control homogenates and subsequent calculation by the method of Cheng and Prusoff (21) yielded a K_i for unlabeled spiroperidol of 0.23 ± 0.07 (n = 2), based on a [^{3}H]apomorphine K_D = 4.44 nM, in good agreement with the K_D = 0.09 nM for [^{3}H]spiroperidol binding to D-2 sites determined above by saturation studies. Hill analysis yielded a pseudo-Hill coefficient of 1.10 ± 0.06, consistent with there being a single homogeneous subset of D-2 binding sites responsible for the phenoxybenzamine-labile portion of [^{3}H] apomorphine high-affinity binding.

Similar results were obtained when unlabeled domperidone was used as the displacing antagonist (Fig. 4B). Again, displacement of [^{3}H]apomorphine by the antago-

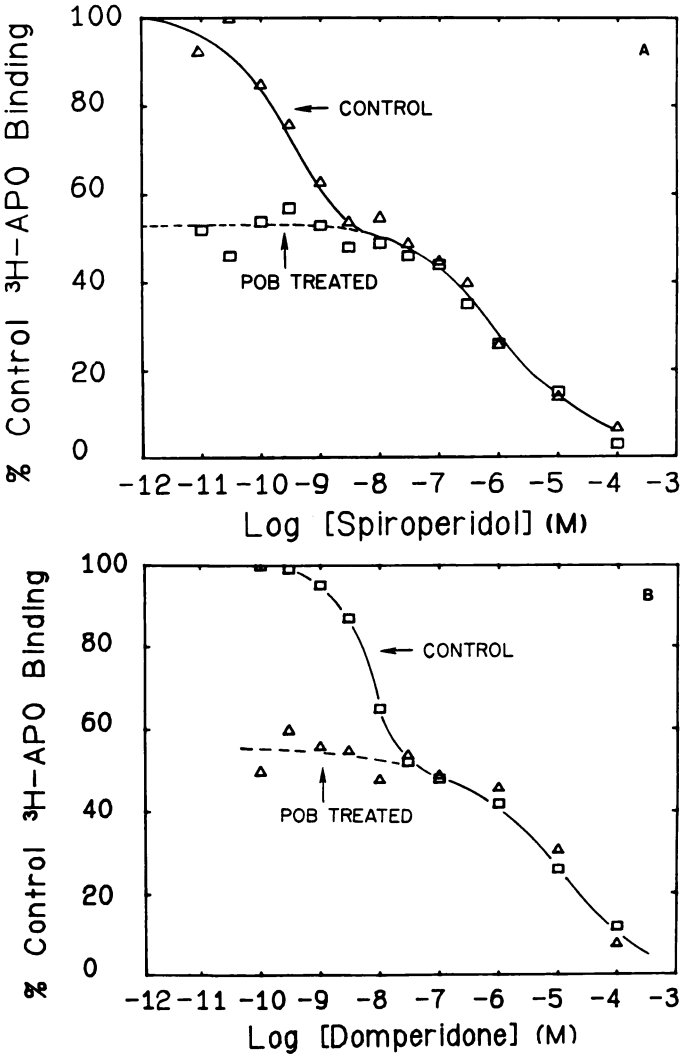

FIG. 4. *Displacement of [^{3}H]apomorphine (^{3}H-APO) binding by spiroperidol and domperidone in control and phenoxybenzamine (POB)-treated homogenates*

Displacement of [^{3}H]apomorphine by spiroperidol (A) and domperidone (B) from control homogenates was biphasic (——). Pretreatment of homogenates with phenoxybenzamine eliminated the high-affinity displacement phase, leaving the low-affinity phase unaffected (- - -). Caudate homogenates were pretreated with 10 μM phenoxybenzamine for 10 min and then thoroughly washed.

A. Displacement by spiroperidol. Various concentrations of unlabeled spiroperidol were added to tubes containing 0.8 nM [^{3}H]apomorphine, tissue sample, and, for nonspecific binding determinations, 10 μM (+)-butaclamol. Results are expressed as the percentage of specific binding to control membranes without displacing drug. Points represent the mean of two separate experiments with standard errors of the mean less than 10%.

B. Displacement by domperidone. Points represent a single experiment performed as in A.

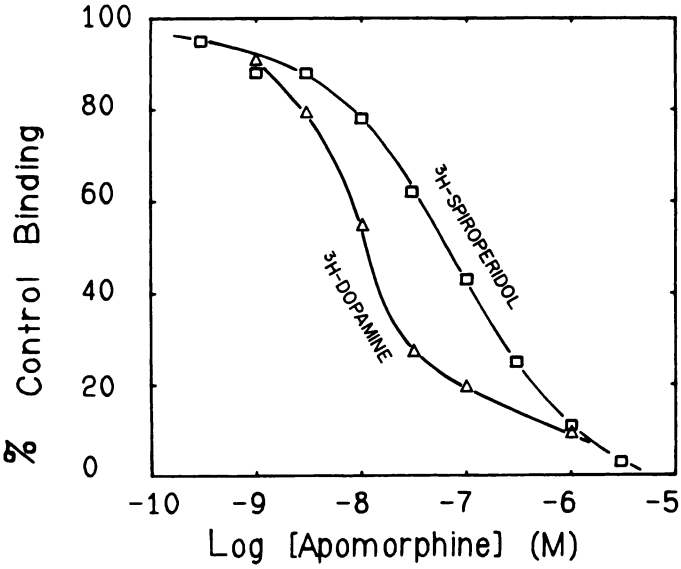

FIG. 5. *Displacement of [^{3}H]spiroperidol and [^{3}H]dopamine by apomorphine*

Various concentrations of unlabeled apomorphine were added to tubes containing 0.1 nM [^{3}H]spiroperidol and 30 nM R41468, or 3 nM [^{3}H]dopamine, tissue sample, and, for the determination of nonspecific binding, (+)-butaclamol. Results are expressed as the percentage of specific binding to control membranes without displacing apomorphine. Note that these homogenates were not pretreated with phenoxybenzamine. Points represent a typical experiment, repeated twice.

nist proceeded in a clearly biphasic curve, with a pseudo-Hill coefficient of about 0.5, consistent with the presence of distinct [^{3}H]apomorphine binding sites with different affinities for domperidone. The K_i of domperidone for the phenoxybenzamine-labile [^{3}H]apomorphine sites, as measured by log-probit plots of the high-affinity phase of displacement and conversion of IC_{50} to K_i, is 4.6 nM, in reasonable agreement with domperidone's affinity of 2.0 nM at [^{3}H]spiroperidol-labeled D-2 sites. This high-affinity displaceable [^{3}H]apomorphine binding was again phenoxybenzamine-labile, whereas that with low affinity for domperidone was not.

As reported previously (12) dopamine was potent in the displacement of [^{3}H]apomorphine from control homogenates, with a K_i of 20 nM. This value is close to the K_D obtained in [^{3}H]dopamine saturations under these conditions (8)[5] and was not significantly changed for homogenates previously treated with phenoxybenzamine to eliminate phenoxybenzamine-labile [^{3}H]apomorphine binding (data not shown).

Displacement of [^{3}H]spiroperidol and [^{3}H]dopamine by apomorphine. If [^{3}H]apomorphine labeled with high affinity only about 7 pmoles/g out of the 9 pmoles/g of [^{3}H]spiroperidol binding sites as suggested by the saturation studies (Fig. 3), it would be anticipated that unlabeled apomorphine would displace only part of [^{3}H]spiroperidol binding with high affinity. This would be expected to produce an over-all apomorphine K_i less than the K_D for [^{3}H]apomorphine and to yield a shallow displacement curve with a pseudo-Hill slope less than 1.0. Indeed, apomorphine displaced [^{3}H]spiroperidol from control homogenates, even in the presence of R41468 to eliminate confounding binding to 5-HT_2 sites, with an IC_{50} of 60 nM, corresponding after conversion (21) to a K_i of 30 nM (Fig. 5). Hill analysis yielded a pseudo-Hill slope of 0.70.

That the phenoxybenzamine-stable [^{3}H]apomorphine binding sites were equivalent to D-3 sites labeled by [^{3}H]dopamine was supported by the potent displacement of [^{3}H]dopamine by apomorphine (Fig. 5). Apomorphine displayed an over-all K_i of 9.5 ± 1.8 nM (n = 2) based on a K_D = 10 nM[5] for [^{3}H]dopamine. This agreed well with the K_D of 4.4 nM for [^{3}H]apomorphine itself. The pseudo-Hill slope of this displacement was approximately 0.8.

Protection of [^{3}H]spiroperidol and [^{3}H]apomorphine binding sites from phenoxybenzamine inactivation by dopaminergic agents. Various agents were included in the tissue preincubation/phenoxybenzamine exposure period to assess the effect of receptor occupancy on phenoxybenzamine inactivation. This co-incubation with a variety of dopaminergic and nondopaminergic compounds produced post-washout protection of [^{3}H]spiroperidol binding in proportion to their affinity for D-2 sites (Fig. 6). Domperidone protection against phenoxybenzamine attack increased smoothly to near-complete protection over a 3-log concentration range with an EC_{50} of 2.7 ± 0.4 nM (n = 4). This value was quite close to the affinity of domperidone for [^{3}H]spiroperidol-labeled D-2 sites (K_i = 2 nM),[5] consistent with an occupancy-mediated mechanism of protection. Dopamine itself displaced a protection EC_{50} of approximately 1 μM, compared with a

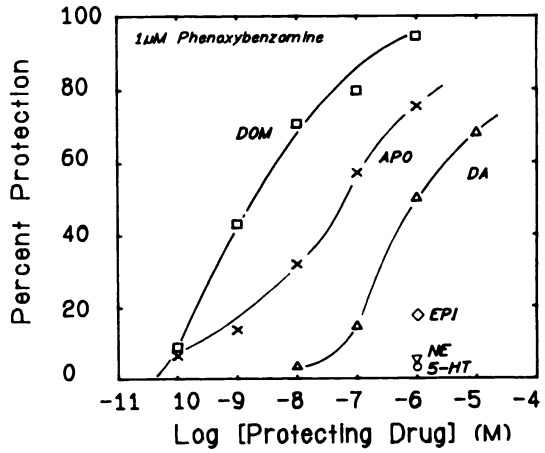

FIG. 6. *Protection of [^{3}H]spiroperidol binding sites by inclusion of various drugs during homogenate phenoxybenzamine exposure*

Domperidone (*DOM*), apomorphine (*APO*), dopamine (*DA*), epinephrine (*EPI*), norepinephrine (*NE*), or serotonin (*5-HT*) were added at various concentrations to homogenate samples. These were preincubated for 10 min at 37°. After addition of 1 μM phenoxybenzamine, they were allowed to incubate for an additional 10 min. The homogenates were then extensively washed to remove both phenoxybenzamine and protecting drug, and assayed for [^{3}H]spiroperidol (0.5 nM) specific binding. Results shown are means of two to five independent determinations with standard error of the mean <15%. *Percent protection* of [^{3}H]spiroperidol binding = $(B_{prot} - B_{POB}/B_{cont} - B_{POB}) \times 100$, where B_{POB} is specific binding after treatment with phenoxybenzamine alone, B_{prot} is that after phenoxybenzamine with protecting agent, and B_{cont} that after neither phenoxybenzamine nor protecting agent, but otherwise processed exactly as the other samples. Inclusion of protecting drug alone without phenoxybenzamine followed by washout had no effect on binding for any of the compounds tested.

[5] M. W. Hamblin and I. Creese, unpublished observations.

K_i of 3.6 μM (22) for D-2 sites. Apomorphine was intermediate in protection potency with an EC_{50} of 60 nM commensurate with its intermediate ability to occupy D-2 sites. Epinephrine conferred much less protection, and norepinephrine and serotonin, which have extremely low affinity for D-2 sites (11), showed no significant protection. Similar results were obtained when [^{3}H]domperidone was used as the ligand (data not shown).

The binding of [^{3}H]apomorphine to its phenoxybenzamine-labile sites could be similarly protected by occupancy with apomorphine, as shown in Fig. 7. When 1 μM phenoxybenzamine was used during the standard 10-min exposure, apomorphine conferred somewhat greater protection of phenoxybenzamine-labile [^{3}H]apomorphine binding sites than of [^{3}H]spiroperidol sites, although this difference did not reach statistical significance.

DISCUSSION

This study exploited the ability of phenoxybenzamine to inactivate selectively and irreversibly D-2 dopamine binding sites labeled by ^{3}H-butyrophenones, but not D-3 dopamine binding sites labeled by [^{3}H]dopamine, to characterize further these receptor binding site subtypes in bovine caudate membranes.

Preincubation of membranes with phenoxybenzamine eliminates high-affinity binding of the D-2-selective ligand [^{3}H]spiroperidol (6). This effect on ^{3}H-butyrophenone binding is rapid in onset ($t_{1/2}$ = 3 min for 3 μM phenoxybenzamine), and requires low concentrations of the alkylating drug. The pseudo-IC_{50} of approximately 1 μM under our conditions in the inhibition of [^{3}H]spiroperidol binding is approximately 100 times lower than that required to inhibit the binding of the D-3-selective ligand [^{3}H]dopamine. Phenoxybenzamine shows pseudo-

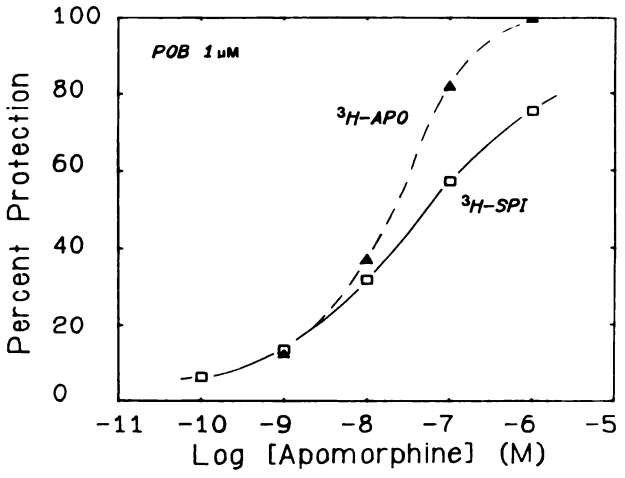

FIG. 7. *Protection of [^{3}H]spiroperidol (^{3}H-SPI) and phenoxybenzamine (POB)-labile [^{3}H]apomorphine (^{3}H-APO) binding sites by unlabeled apomorphine*

The [^{3}H]spiroperidol curve is reproduced here from Fig. 6 for comparison. Results are expressed as *percent protection* as defined in Fig. 6 of [^{3}H]spiroperidol (0.5 nM) and phenoxybenzamine-labile [^{3}H]apomorphine (0.8 nM) specific binding by various concentrations of apomorphine included during homogenate phenoxybenzamine (1 μM) treatment. Phenoxybenzamine-labile [^{3}H]apomorphine specific binding sites were defined as those sites eliminated by 10 μM phenoxybenzamine preincubation. Each point represents the mean of two to four independent determinations ± standard error of the mean.

IC_{50} values for the binding of [^{3}H]haloperidol and [^{3}H]domperidone, two ligands thought to label the same site as that labeled by [^{3}H]spiroperidol (11, 16), quite close to that for [^{3}H]spiroperidol, indicating that the phenoxybenzamine effect is not the idiosyncratic property of a single ligand. The slightly greater resistance to phenoxybenzamine attack noted for [^{3}H]spiroperidol binding relative to that of [^{3}H]domperidone may reflect a resistance to alkylation of the few 5-HT_2 sites labeled by [^{3}H]spiroperidol.

Phenoxybenzamine-mediated inhibition is site-directed, as shown by the protection afforded by receptor occupancy, whether by agonists or antagonists. This inhibition is mediated predominantly via a decrease in B_{max}. There is also a small accompanying decrease in the affinity of residual sites for [^{3}H]spiroperidol, perhaps reflecting nonspecific membrane-perturbing effects. This decrease in B_{max} and the irreversible nature of the inhibition with respect to repeated washings (6, 23) strongly suggest that phenoxybenzamine produces its inhibition of [^{3}H]spiroperidol binding by alkylation of the receptor moiety, as is believed for its actions at the *alpha*-adrenergic receptor (24).

Treatment with phenoxybenzamine sufficient to eliminate almost all (95%) [^{3}H]spiroperidol binding sites also inactivates 40–50% of [^{3}H]apomorphine sites, or about 7 pmoles/g of tissue. This value is slightly less than the total number of D-2 sites labeled by [^{3}H]spiroperidol (9 pmoles/g). The affinity of unlabeled spiroperidol for these phenoxybenzamine-labile [^{3}H]apomorphine sites (K_i = 0.2 nM) is very close to the affinity of [^{3}H]spiroperidol for its binding sites as established in saturation studies (K_D = 0.09 nM). Thus, [^{3}H]apomorphine labels a major portion, but not all, of the D-2 sites labeled by [^{3}H]spiroperidol. This conclusion is further supported in that the two methods of assaying the D-2 sites, by determining either [^{3}H]spiroperidol binding or "phenoxybenzamine-labile" [^{3}H]apomorphine binding, yield identical results for both phenoxybenzamine dose response and for protection mediated by occupancy. The affinity of apomorphine for these sites as determined directly by saturation studies (K_D = 4.4 nM) does not agree well with the affinity determined by displacement of [^{3}H]spiroperidol (K_i = 30 nM). This may result from the presence of a minor fraction (about 2 pmoles/g) of D-2 sites having much lower apomorphine affinity. Only a fraction of the [^{3}H]spiroperidol binding is displaced within that part of the shallow (pseudo-Hill slope = 0.7) curve near the [^{3}H]apomorphine K_D. These findings are thus similar to those for [^{3}H]spiroperidol binding to D-2 sites in the anterior pituitary. In this tissue [^{3}H]apomorphine also labels only a fraction of D-2 receptors with high affinity, and unlabeled apomorphine displaces [^{3}H]spiroperidol from D-2 sites with a pseudo-Hill coefficient of less than 1.0 (2, 10). These latter observations are thought to reflect the existence of two states of a single pituitary D-2 receptor, with respective high and low affinities for agonists, but with equal affinities for antagonists. The above data, in addition to the pharmacological similarities between brain and pituitary D-2 receptors (2, 11) suggest that caudate D-2 receptors may also exist in two states with different agonist affinities. The existence of distinct high- and low-affinity states of the better-characterized *beta*-

adrenergic receptor binding sites accounts for the lowered affinity and decreased pseudo-Hill coefficient in agonist displacement of labeled antagonists in this system (25, 26).

[^{3}H]Apomorphine also labels an additional 8 pmoles/g of caudate binding sites which are phenoxybenzamine-stable and have very low (1 μM) affinity for spiroperidol and domperidone, but high (20 nM) affinity for dopamine. As Scatchard plots of control [^{3}H]apomorphine saturations are linear, and as the K_D of the phenoxybenzamine-stable [^{3}H]apomorphine binding sites is the same as that for control [^{3}H]apomorphine binding, the affinity of [^{3}H]apomorphine for these two dopaminergic binding site subtypes must be approximately equal at about 4 nM. This value is also in good agreement with the affinity of apomorphine's for D-3 sites labeled by [^{3}H]dopamine (K_i = 9.5 nM). This demonstrates that [^{3}H]apomorphine also labels D-3 sites, in addition to D-2 sites. These findings are consistent with those reported by Sokoloff *et al.* (27), who found two [^{3}H]apomorphine binding sites in rat striatum, one with high domperidone affinity, removed by kainic acid lesion, and another with low domperidone affinity, not removed by kainic acid lesion. Binding at D-3 sites, as shown by displacement of phenoxybenzamine-stable [^{3}H]apomorphine binding by spiroperidol and domperidone and by the binding of [^{3}H]dopamine itself, remains unchanged by 10 μM phenoxybenzamine treatment.

Thus, phenoxybenzamine distinguishes two pharmacologically separate dopaminergic binding sites: D-2 sites with high (nanomolar) affinity for [^{3}H]spiroperidol, and D-3 sites with high affinity for [^{3}H]dopamine. [^{3}H]Apomorphine labels, at least to a major degree, both subsets of binding sites. Furthermore, [^{3}H]dopamine labels some sites distinct from those labeled by [^{3}H]apomorphine. Although the total number of [^{3}H]dopamine-specific binding sites is approximately equal to that of [^{3}H]apomorphine (12),[5] some [^{3}H]apomorphine binding but not [^{3}H]dopamine binding is to [^{3}H]butyrophenone binding sites. Thus, the number of sites "shared" by [^{3}H]dopamine and [^{3}H]apomorphine cannot exceed about 60% of the total for each. This third [^{3}H]dopamine binding site labeled by [^{3}H]dopamine, possessing much lower affinity for apomorphine and butyrophenones, may account for the pseudo-Hill slope <1 observed in apomorphine displacement of dopamine and has yet to be investigated in isolation.

Marchais and Bockaert (23) demonstrated that phenoxybenzamine treatment of rat striatal membranes sufficient to eliminate [^{3}H]spiroperidol binding leaves 35% of dopamine-stimulated adenylate cyclase activity intact. Thus, they suggested, dopamine receptors labeled with [^{3}H]spiroperidol are not cyclase-linked. We found that sites labeled by [^{3}H]flupentixol, a ligand which is partially selective for D-1 dopamine receptors linked to adenylate cyclase (4, 28) were somewhat more resistent than [^{3}H]spiroperidol sites to phenoxybenzamine treatment, consistent, in general, with the above view. It should also be noted that 85% of [^{3}H]dopamine binding remains intact after this pretreatment, suggesting that the D-3 sites labeled by this ligand are not adenylate cyclase-linked.

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