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CHARACTERIZATION OF BINDING SITES FOR [^{3}H]SPIROPERIDOL

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Abstract—Experiments were designed to investigate the biochemical properties of binding sites for [^{3}H]spiroperidol ([^{3}H]SPD) solubilized from canine caudate and to define the effect of detergent on the binding of the radioligand. Extraction of canine caudate with 0.75–1.0% digitonin was found to generate the maximum yield of binding sites for [^{3}H]SPD while minimizing extraction of membrane proteins. Although binding sites were solubilized with 1.0% digitonin, a 10-fold reduction in detergent concentration was necessary to achieve maximal binding of [^{3}H]SPD. The rank order of affinity for agonists and antagonists was consistent with the pharmacologic properties of the D_2 subtype of the dopamine receptor. However, the binding of antagonists was found to be complex. Studies with some preparations of pooled canine caudate resulted in competition curves for the D_2-selective antagonists domperidone and sulpiride that best fit a single-site model. Other preparations exhibited biphasic inhibition curves with these antagonists. The class of binding sites for [^{3}H]SPD with low affinity for D_2-selective antagonists constituted as much as 30–40% of the binding sites. Enrichment of solubilized binding sites for [^{3}H]SPD was achieved by size exclusion HPLC followed by adsorption to DEAE-Sephadex and elution with buffer of increasing ionic strength. Enrichment of binding sites was accompanied by a decrease in the affinity of solubilized sites for [^{3}H]SPD.

Two distinct subtypes of dopamine (DA†) receptors have been identified on the basis of their opposing effects on the adenylate cyclase system. Kebabian and Calne [1] suggested that the DA receptors linked to the stimulation of adenylate cyclase be classified as D_1 receptors, while the DA receptors that do not increase enzymatic activity be classified as D_2 receptors. The apparent affinity of agonists for D_2 receptors labeled with [^{3}H]spiroperidol ([^{3}H]SPD) is decreased when assays are carried out in the presence of guanine nucleotides [2]. This result suggests that D_2 receptors modulate adenylate cyclase through a guanine nucleotide-binding protein [3–5]. Direct demonstration of the inhibition of adenylate cyclase by D_2 receptors has been obtained in studies with tissue from rat pituitary [6] and striatum [7].

The availability of high-affinity, D_2-selective antagonists such as domperidone, sulpiride and the butyrophenones haloperidol and SPD has made it possible to carry out preliminary studies on the structural and functional properties of D_2 receptors. Studies of detergent solubilization of DA receptors have focused primarily on the D_2 subtype, with the binding of [^{3}H]SPD being used to quantitate the yield of solubilized receptor. Protocols for the solubilization of D_2 receptors from caudate have been reported using a variety of detergents, either in the absence or presence of high concentrations (>0.75 M) of NaCl [8–15]. Solubilization of binding sites for [^{3}H]SPD has also been reported using the anterior pituitary, a tissue that appears to be devoid of D_1 receptors [16–18].

Several laboratories have used digitonin to solubilize D_2 receptors. However, even when extraction protocols were similar, the binding of [^{3}H]SPD to solubilized preparations has been measured with different concentrations of digitonin in the binding assay. In this study we have examined the effect of digitonin on the extraction of binding sites for [^{3}H]SPD from caudate tissue, as well as the effect of this detergent on the binding of [^{3}H]SPD to solubilized preparations. The binding of [^{3}H]SPD was found to be dependent on the concentration of both detergent and protein in the binding assay. Increasing the concentration of digitonin in the binding assay decreased the binding of [^{3}H]SPD. The rank orders of affinity of agonists and antagonists at DA and serotonin receptors were consistent with the pharmacologic properties of the D_2 subtype of DA receptors. While inhibition of the binding of [^{3}H]SPD by agonists modelled as a single site, the binding of antagonists to solubilized preparations was found to be more complex than previously reported. An enrichment of solubilized binding sites for [^{3}H]SPD was achieved using size exclusion HPLC and DEAE-Sephadex ion-exchange chromatography. Enrichment of binding sites was accompanied by a decrease in the affinity of the binding sites for [^{3}H]SPD.

MATERIALS AND METHODS

Materials. Ligands and reagents were obtained from the following suppliers: [^{3}H]SPD, New England Nuclear (Boston, MA) or Amersham (Arlington

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† Abbreviations: DA, dopamine; [^{3}H]SPD, [^{3}H]spiroperidol; APO, apomorphine; NPA, *N*-propylnorapomorphine; 6,7-ADTN, 2-amino-6,7-dihydroxytetrahydronaphthalene; and PEG, polyethylene glycol, PMSF, phenylmethylsulfonyl fluoride.

Heights, IL); (+)-butaclamol, (−)-butaclamol, *N*-propylnorapomorphine (NPA) and 2-amino-6,7-dihydroxytetrahydronaphthalene (6,7-ADTN), Research Biochemicals Inc. (Wayland, MA); apomorphine (APO), dopamine-HCl, digitonin, bovine gamma globulin, polyethylene glycol (PEG), aprotinin, leupeptin, soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (PMSF), Sigma Chemical Co. (St. Louis, MO); domperidone, ketanserin and pipamperone, Janssen Pharmaceuticals (Beerse, Belgium); sulpiride, Ravizza Pharmaceuticals (Milan, Italy); and A-50 DEAE-Sephadex, Pharmacia Fine Chemicals (Piscataway, NJ).

Radioligand binding assays. Frozen dog brains (Pel Freeze Biologicals; Rogers, AR) were received on dry ice and kept at −70°. For each tissue preparation, ten to twenty-five brains were thawed overnight on ice. The head of the caudate was dissected and kept on ice in 100 mM Tris–HCl, pH 7.5, with 2 mM $MgCl_2$ and 150 mM NaCl. The wet weight of the canine caudate was approximately 1.5 g per brain. Pooled tissue was homogenized with a Polytron homogenizer (setting 6–7) for 5–10 sec and centrifuged at 14,000 *g* for 10 min at 4°. Pellets were resuspended in the same buffer (10 ml/brain), homogenized, and frozen in 5.0-ml aliquots at −70°.

In studies of the binding of [^{3}H]SPD to membranes, homogenates were thawed on ice, centrifuged at 14,000 *g* for 10 min at 4° and resuspended in 50 mM Tris–HCl, pH 7.5, with 2 mM $MgCl_2$ and 150 mM NaCl. Incubations were carried out in a final volume of 2.0 ml for 12–16 hr at 4°. These conditions were selected to facilitate direct comparison with results obtained when detergent-solubilized preparations were used. Nonspecific binding was defined by including 2 μM (+)-butaclamol in duplicate sets of assays. Assays were terminated by the addition of 5 ml of 10 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, followed immediately by filtration over a glass-filter disc (Schleicher & Schuell, No. 30). Each filter was then washed twice with 5 ml of the above buffer. Buffers for the filtration and washing were kept on ice.

In studies of the binding characteristics of solubilized receptors, homogenates were thawed on ice and centrifuged for 10 min at 14,000 *g*. Membranes were resuspended and homogenized in 5.0 ml per caudate equivalent of 100 mM Tris–HCl, pH 7.5, containing 4 mM $MgCl_2$ and 300 mM NaCl. An equal volume of a 2.0% solution of digitonin in water was then added. The preparation was stirred vigorously and kept at 0° for 60 min. Tissue not solubilized by detergent was removed by centrifugation at 100,000 *g* for 60 min at 4°. The supernatant fraction was kept at 0° until added to the binding assay.

Digitonin was prepared as a 5.0% stock solution in water. Since the purity of the digitonin was approximately 80%, the 5.0% stock solution consisted of 6.25 g of digitonin in 100 ml of water. Although digitonin was dissolved by stirring in a boiling water bath, storage of the solution at 4° often resulted in the formation of a precipitate. Several different lots of digitonin were used to prepare stock solutions. Those lots which contained the least amount of precipitate were used for tissue solubilization. The stock solutions of digitonin were filtered through Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) prior to use.

A protein precipitation assay [19] using PEG was used to measure the binding of [^{3}H]SPD to digitonin-solubilized tissue preparations. Test tubes (105 × 16 mm, Sarstedt, No. 55.538) containing 50 mM Tris–HCl, pH 7.5, with 2 mM $MgCl_2$ and 150 mM NaCl and the appropriate concentration of [^{3}H]SPD (in the presence or absence of a competing ligand) were kept on ice. An aliquot of the supernatant fraction from caudate tissue extracted with digitonin was added to each tube. The solutions were thoroughly mixed and the reaction mixture was incubated at 4° for 12–16 hr. Solubilized proteins were precipitated by adding an aliquot (0.1 × the volume of the reaction mixture) of 10 mg/ml of bovine gamma globulin in 50 mM Tris–HCl, pH 7.5, followed by an aliquot (0.5 × the volume of the reaction mixture) of 30% PEG (approximate molecular weight of 8000) in 50 mM Tris–HCl, pH 7.5. The mixture was incubated for 10 min at 0°, and 5.0 ml of a solution containing 10% PEG (w/v) in the same buffer was added to each tube. The contents were immediately filtered on a glass-filter disc (Schleicher & Schuell, No. 30) and washed twice with 5.0 ml of a solution containing 10% PEG. Radioactivity was determined using a toluene-based fluor containing 4.0 g/l of 2a70 (Research Products International, Mt. Prospect, IL). When a charcoal adsorption assay was used to measure the binding of [^{3}H]SPD to the solubilized preparations, a modification of the method of Ilien *et al.* [20] was used. The modifications included a 12- to 16-hr incubation at 4° in a final volume of 1.0 ml. After centrifugation, 0.8 ml of supernatant fraction was used to determine the amount of radioligand remaining in the supernatant. Nonspecific binding was defined by the inclusion of 2 μM (+)-butaclamol in the assay.

Data analysis of direct and indirect binding. Values for equilibrium dissociation constants (K_d) and maximum number of binding sites (B_{max}) were obtained using unweighted linear regression analysis of data transformed according to the method of Scatchard [21]. Competition curves were modelled for one site using the following equation:

$$B = \frac{B_o}{1 + (L/IC_{50})} + B_{ns} \qquad (1)$$

In equation 1, B is the amount of radioligand bound to the tissue, B_o is the amount of ligand bound in the absence of competing ligand, L is the concentration of the competing ligand, IC_{50} is the concentration of competing ligand that inhibits 50% of total specific binding, and B_{ns} is the nonspecific binding of the radioligand. Curve fitting was done by nonlinear least squares regression analysis according to the method of Marquardt and Levenberg (see Ref. 22). The analysis was performed by the mathematical modelling program NEWFITSITES available on the NIH-sponsored PROPHET system. The values for B_{ns} and B_o were constrained using experimentally derived values.

Competition data were also fit to the following

two-site model:

$$B = \frac{B_1}{1 + (L/IC_{50}{}^1)} + \frac{B_2}{1 + (L/IC_{50}{}^2)} + B_{ns} \quad (2)$$

In equation 2, B_1 and B_2 are the amounts of radioligand bound to each site in the absence of competing ligand and $IC_{50}{}^1$ and $IC_{50}{}^2$ are the IC_{50} values of the competing ligand for each site. Curve fitting was performed by the NEWFITSITES program as described above. Improvement of fit of the two-site model compared to the one-site model was determined with a partial F-test on the residual sum of squares [23]. The IC_{50} values were transformed to $K_{0.5}$ values using the method of Cheng and Prusoff [24].

The protein concentration of membrane and solubilized preparations was determined by the method of Bradford [25] using either bovine gamma globulin or bovine serum albumin as standards. When the concentration of detergent-solubilized protein was to be determined, the samples for the standard curve included an appropriate concentration of digitonin.

Size-exclusion HPLC and ion-exchange chromatography. Size-exclusion HPLC was carried out with a TSK-2-3000 precolumn (7.5 mm × 10 cm) with tandem TSK-4000 and TSK-3000 columns (7.5 mm × 30 cm). The buffer system was 100 mM Tris–HCl, pH 7.5, containing 0.1% digitonin. Buffers were filtered (type HA 0.45 μm, Millipore) and degassed prior to use. The columns and buffer were kept on ice. The flow rate of the system was maintained at 1.0 ml/min using a Waters HPLC pump (model 6000A). Fractions were collected every 0.5 min. Ultraviolet absorption at 280 nm was recorded with a Waters 440 Absorption Detector. Estimates of the molecular size of the protein–detergent complex were obtained graphically by comparing the peak binding activity to the elution profiles of a series of proteins of known molecular weight chromatographed under the same conditions. The molecular weight standards used were human IgM (900,000 daltons), ferritin (540,000 daltons), catalase (240,000 daltons) and aldolase (158,000 daltons). A 250-μl aliquot from each fraction was placed into a binding tube and kept on ice. Each aliquot was diluted with 200 μl of cold 300 mM NaCl solution containing 0.1% digitonin. For the binding of [^{3}H]SPD, the final concentration of radioligand was 2.0 nM and the final volume was 0.5 ml. Nonspecific binding was determined by including 2.0 μM (+)-butaclamol in the binding assay. Samples were incubated overnight at 4°, and the PEG precipitation method was used to quantitate the binding of [^{3}H]SPD.

DEAE-Sephadex was equilibrated in 50 mM Tris–HCl buffer, pH 7.5, containing 10 mM EDTA, 0.1% digitonin and 150 mM NaCl at 4°. After application of the protein sample, the column was washed with four to five times the column volume of the equilibration buffer. Adsorbed protein was eluted with a linear 150 to 500 mM NaCl gradient in the equilibration buffer. The binding of [^{3}H]SPD in each fraction was measured by incubating 0.25 ml of each fraction with [^{3}H]SPD (2.0 nM) in a final volume of 0.3 ml overnight at 4°. The PEG precipitation assay was used to quantitate the binding of [^{3}H]SPD.

RESULTS

Effect of digitonin on the binding of [^{3}H]*SPD to detergent-solubilized preparations of canine caudate.* Several laboratories have reported that binding sites for [^{3}H]SPD can be solubilized from canine caudate using 1.0% digitonin. However, a variety of conditions have been used to measure the binding of [^{3}H]SPD in the solubilized preparations. Therefore, both the detergent extraction protocol and the effect of detergent on the binding were examined. A constant amount of homogenate of canine caudate tissue was extracted with increasing concentrations of digitonin. After centrifugation, the protein concentration and the binding of [^{3}H]SPD in the supernatant fraction were measured. Over a range of concentrations of digitonin from 0 to 2.0%, the amount of protein in the supernatant fraction increased. Approximately 90% of the membrane-associated protein was extracted when 2.0% digitonin was used. While total extracted protein increased, the apparent yield of solubilized binding sites for [^{3}H]SPD was maximal and constant over a range of concentrations of digitonin from 0.75 to 2.0% (Fig. 1A). In determining the effectiveness of solubilization protocols, the following experimental manipulations were performed: (1) pellets obtained after centrifugation of detergent-extracted caudate tissue were washed twice with buffer (without detergent) to reduce the final concentration of digitonin in the binding assay; and (2) the final concentration of digitonin in the binding assay of solubilized preparations was adjusted to 0.1%. The digitonin concentration was adjusted to 0.1% because the binding of [^{3}H]SPD to solubilized binding sites decreased as the concentration of digitonin in the binding assay increased (Fig. 1B). The maximum amount of binding of ligand was observed when the percentage of digitonin in the binding assay was 0.075–0.1%. When the final concentration of digitonin in the binding assay was 0.05%, the amount of specific binding was often reduced, suggesting that a minimum amount of detergent may be needed to maintain the stability of the solubilized protein. Results similar to those shown in Fig. 1B were obtained using a charcoal ligand-adsorption binding assay (data not shown). Therefore, the observed digitonin-dependent decrease in the specific binding of [^{3}H]SPD was not due to interference with the precipitation of protein by PEG. The yield of solubilized sites for [^{3}H]SPD from canine caudate using 1.0% digitonin was 15–18%.

Pharmacologic characterization of the solubilized binding sites for [^{3}H]*SPD.* The pharmacologic properties of the binding sites for [^{3}H]SPD were investigated using agonists and antagonists known to show selectivity for DA and serotonin receptors. Nonspecific binding was defined experimentally by including 2 μM (+)-butaclamol in duplicate binding assays. The average $K_{0.5}$ values for the binding of agonists and antagonists to digitonin-solubilized extracts of canine caudate were calculated from competition curves such as those shown in Figs. 2 and

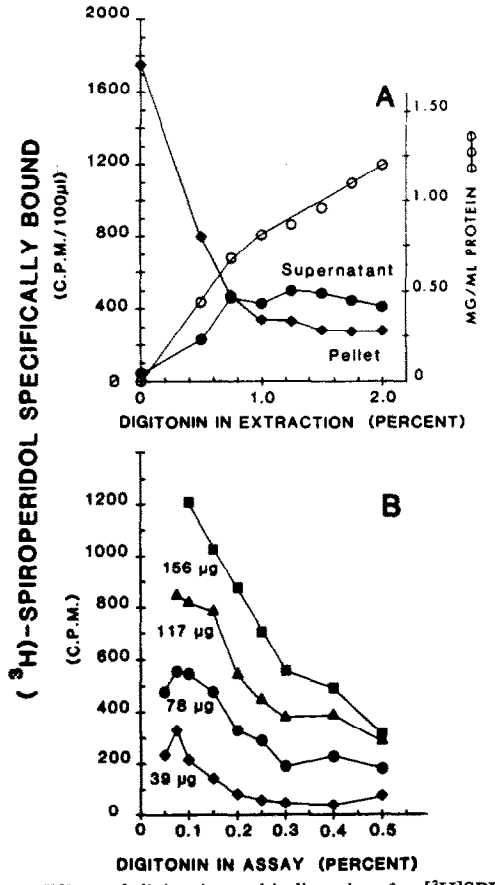

Fig. 1. Effect of digitonin on binding sites for [^{3}H]SPD. (A) Homogenates of canine caudate were extracted with 0–2.0% (w/v) digitonin at a final protein concentration of 1.2 mg/ml. The extraction buffer contained 50 mM Tris–HCl, 2 mM $MgCl_2$ and 150 mM NaCl at pH 7.5. After extraction for 1.0 hr at 0° the samples were centrifuged at 100,000 *g* for 1.0 hr at 4°. Supernatant fractions were kept on ice. Pellets were washed twice in the same buffer without detergent and resuspended in a volume of buffer equivalent to the original extraction volume. Binding of [^{3}H]SPD was measured using a 0.1-ml aliquot of either the supernatant fraction (●) or the resuspended pellet (◆) in a final volume of 2.0 ml. An aliquot of a 5% solution of digitonin was added such that the final concentration of digitonin in the assay was 0.1%. Samples were incubated at 4° for 14 hr with [^{3}H]SPD (2.0 nM). In this experiment, 1000 cpm represents 55 fmol of ligand specifically bound. Protein concentrations in the supernatant fraction (○) were determined using bovine serum albumin as a standard. Each point is the average of quadruplicate determinations, and the data shown are representative of results in four similar experiments. (B) Homogenates of canine caudate were extracted with 1.0% digitonin at a final concentration of protein of 1.3 mg/ml. After centrifugation at 100,000 *g* for 60 min, aliquots of the supernatant fraction (0.78 mg protein/ml) were used to measure the binding of [^{3}H]SPD (2.0 nM). The final volume of the binding assay was 2.0 ml. The percent of digitonin refers to the final percentage in the binding assay. The PEG precipitation protocol was used. Each point represents the average of triplicate determinations. The amount of supernatant protein used for each curve was as follows: 39 µg protein (◆); 78 µg protein (●); 117 µg protein (▲); and 156 µg protein (■). In this experiment, 1000 cpm represents 55 fmol of ligand specifically bound. Protein concentrations were determined using bovine serum albumin as a standard. Data shown are representative of results from three similar experiments.

3 (Table 1). Nonlinear regression analysis of the competition curves using agonists indicated that a single-site model adequately fit the data for all preparations of solubilized binding sites (Fig. 2). The order of potency for the inhibition of binding of [^{3}H]SPD by agonists was NPA > APO = 6,7-ADTN > DA. Serotonin had almost no effect on the binding of [^{3}H]SPD to these solubilized preparations. This order of potency is consistent with previously reported results obtained with digitonin-solubilized binding sites for [^{3}H]SPD from canine caudate [26, 27], as well as from bovine and porcine pituitary [17, 18]. Although agonists compete for the binding of [^{3}H]SPD in solubilized preparations, the high-affinity state associated with ternary complex formation was not observed. While agonist competition curves were best fit to a one-site model, similar competition curves with antagonists were more complex than previously reported [8, 9, 26]. Computer analysis of competition curves using antagonists generally indicated a better fit for two sites than for one. The smaller class of sites, representing from 15 to 30% of the total number of sites, consistently had a lower affinity for antagonists at DA receptors than did the larger class of sites. However, different preparations of pooled canine caudates had varying proportions of sites with high and low affinities for antagonists. Panels A and B of Fig. 3 show the inhibition curves obtained with two D_2-selective antagonists, domperidone and sulpiride, for two different preparations of solubilized canine caudate. The data for one preparation were best fit to a one-site model for each antagonist tested. Other preparations exhibited markedly biphasic inhibition curves for antagonists and the competition data fit better to a two-site model. Variability in the proportion of high- and low-affinity binding sites was observed with different preparations of pooled canine caudates. Similar proportions of high- and low-affinity sites were observed for solubilized and mem-

Table 1. Dissociation constants for the inhibition of the binding of [^{3}H]SPD by agonists and antagonists

	$K_{0.5}$ (nM)
Antagonists	
Ketanserin	>10,000
Pipamperone	2,220 ± 600
Sulpiride	7.7 ± 4.2
(+)-Butaclamol	2.6 ± 0.7
Domperidone	1.2 ± 0.3
Agonists	
Serotonin	33,025 ± 5,896
Dopamine	4,255 ± 369
6,7-ADTN	326 ± 12
Apomorphine	217 ± 12
N-Propylnorapomorphine	35 ± 16

Values for $K_{0.5}$ were calculated from competition curves including those shown in Figs. 2 and 3. The values shown (mean ± SEM) are the averages obtained in studies carried out with three preparations of solubilized canine caudate. For some preparations, the computer analysis indicated a multiple-site model. In this case, the IC_{50} value from the major component was used to calculate $K_{0.5}$.

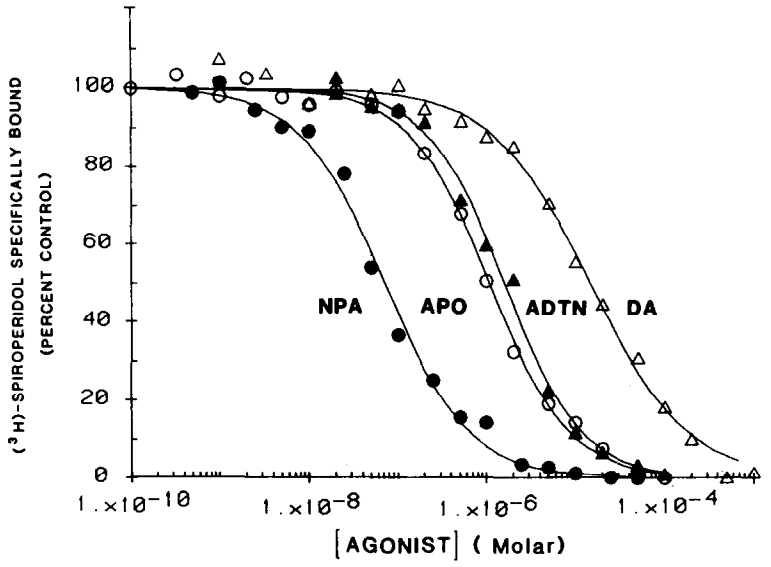

Fig. 2. Inhibition of the binding of [^{3}H]SPD by agonists. The inhibition of the binding of [^{3}H]SPD to solubilized preparations by the agonists *N*-propylnorapomorphine (NPA) (●), apomorphine (APO) (○), 2-amino-6,7-dihydroxytetrahydronaphthalene (6,7-ADTN) (▲) and dopamine (DA) (△) is shown. The curves were best fit to a one-site model. The concentration of [^{3}H]SPD was 1.5 nM, and specific binding was 1150 cpm in the absence of a competing drug. The results shown are representative of four similar experiments.

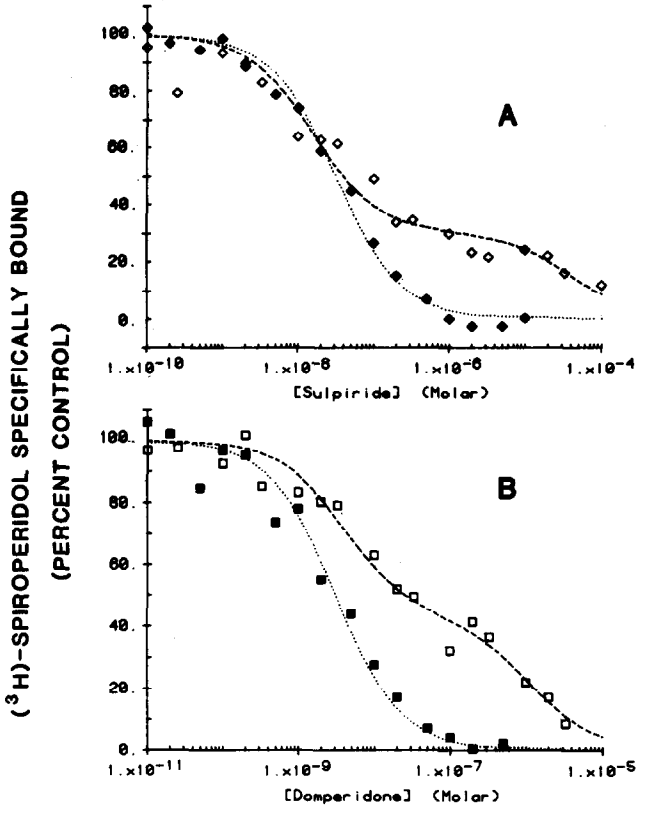

Fig. 3. Inhibition of the binding of [^{3}H]SPD by antagonists. The inhibition of the binding of [^{3}H]SPD by the D_2-selective antagonists sulpiride (A) and domperidone (B) in two preparations of digitonin-solubilized canine caudate is shown. With some preparations of solubilized canine caudate, the inhibition curves with antagonists were best fit to a one-site model (◆, ■). Other preparations exhibited more complex binding properties. Some of the competition curves for domperidone and sulpiride were best fit to a two-site model (◇, □) in which the higher-affinity component constituted 60% (domperidone) and 70% (sulpiride) of the binding sites. The data shown represent the extremes in the variations observed with studies carried out using ten different preparations of pooled canine caudate.

brane-associated sites in a given preparation. The proportion of sites demonstrating low affinity for the D_2 antagonists varied from 0 (i.e. a one-site fit) to 40% for the preparation with the highest percentage of low-affinity component. Biphasic competition curves for solubilized preparations were observed when protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, 10 μg/ml PMSF and 10 mM EDTA) were included in buffers used for the dissection of tissue and for the binding assays.

The inclusion of 10^{-6} M ketanserin (an antagonist selective for the S_2 subtype of the serotonin receptor) was found to decrease the proportion of sites with low affinity for D_2 antagonists. Panels A and B of Fig. 4 show the effect of including a 200-fold molar excess of ketanserin on the binding of [^{3}H]SPD to the two preparations characterized by the competition experiments shown in Fig. 3, A and B. Ketanserin caused only a small decrease in the B_{max} in the preparation in which all of the sites had a high affinity for domperidone and for sulpiride (Fig. 4A). By contrast, in the preparation in which a high percentage of the sites had a low affinity for domperidone and for sulpiride, the addition of ketanserin caused a marked decrease in the density of binding sites for [^{3}H]SPD (Fig. 4B). The results shown in Fig. 3 are the extremes in the variability observed in ten preparations of canine caudate membranes.

Enrichment of digitonin-solubilized binding sites for [^{3}H]*SPD.* Several protocols for the adsorption and elution of solubilized binding sites for [^{3}H]SPD from DEAE-Sephadex were examined. The application of sample in 150 mM NaCl followed by elution with a NaCl gradient from 150 to 500 mM was found to be optimal. Concentrations of digitonin from 0.1 to 1.0% (i.e. after or before size-exclusion HPLC) in the sample did not appear to alter the adsorption of binding sites to the resin. The elution of binding sites for [^{3}H]SPD was measured using the PEG protein precipitation binding assay with 2.0 nM [^{3}H]SPD. However, the elution profile of binding sites for [^{3}H]SPD underestimates the true recovery. The K_d value for the binding of [^{3}H]SPD to sites that adsorbed to DEAE-Sephadex and were eluted with NaCl was found to be 3 to 4-fold higher than that of the solubilized preparations (Table 2). When a correction was made for the decreased K_d value for the DEAE-Sephadex-eluted binding sites, the estimated yield was 80%. The elution profiles for total protein and for the binding sites for [^{3}H]SPD using sequential size-exclusion HPLC (Fig. 5A) followed by adsorption to DEAE-Sephadex and elution with a linear NaCl gradient (Fig. 5B) were determined. Binding sites for [^{3}H]SPD consistently eluted in the included volume of the tandem TSK-3000 and TSK-4000 size-exclusion HPLC columns (Fig. 5A). Fractions containing binding sites for [^{3}H]SPD had an elution time that was earlier than the elution of catalase but later than that of ferritin. Binding sites for [^{3}H]SPD obtained after chromatographic separation of crude solubilized preparations on size-exclusion HPLC columns and DEAE-Sephadex maintained the same rank orders of potency for the binding of agonists and antagonists as was seen in studies of membranes prepared from canine caudate.

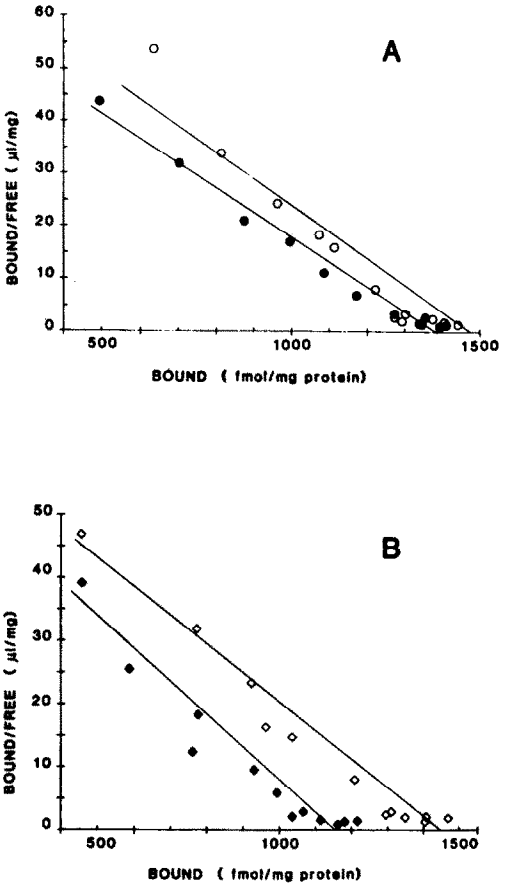

Fig. 4. Binding of [^{3}H]SPD to canine caudate membranes in the presence or absence of an antagonist for the S_2 serotonin receptor. The binding of [^{3}H]SPD to canine caudate membrane preparations (44 μg protein per assay) used for solubilization and competition curves shown in Fig. 3 was studied in the presence (●, ◆) or absence (○, ◇) of ketanserin. The concentration of [^{3}H]SPD in the binding assay ranged from 0.02 to 3.0 nM. When ketanserin was present, its concentration was 200 times that of [^{3}H]SPD. The calculated K_d value for all four experiments was 0.04 nM with linear correlation coefficients from 0.94 to 0.98. The membrane preparation shown in Fig. 4A was used for solubilization and for the monophasic competition curves, shown in Fig. 3, A and B. The decrease in the value for B_{max} in the presence of ketanserin was 7%. The membrane preparation shown in Fig. 4B was similarly used for solubilization and for the biphasic competition curves shown in Fig. 3, A and B. The decrease in the value for B_{max} in the presence of ketanserin was 17.5%.

DISCUSSION

The goal of these studies was to investigate the diversity of the population of solubilized caudate binding sites for [^{3}H]SPD and to understand the effect of detergent on the interaction between the binding sites and the radioligand to establish a rational approach to the purification of D_2 receptors. The experiments were designed to (1) investigate the effect of digitonin on both the extraction of binding sites for [^{3}H]SPD from caudate tissue and the binding

Table 2. Affinity of preparations of canine caudate for [^{3}H]SPD

Tissue preparation	K_d (nM)	B_{max} (fmol/mg protein)
Membrane associated	0.04 ± 0.02	732 ± 176
Detergent-solubilized	0.5 ± 0.1	343 ± 56
Detergent-solubilized and DEAE-Sephadex-enriched	1.8 ± 0.3	1089 ± 218

Scatchard analysis was used to determine the affinity of the receptor for [^{3}H]SPD and the density of binding sites (B_{max}). All assays of solubilized extracts were carried out in the presence of 0.1% digitonin. Protein concentrations were determined using the Bradford protein assay [25] with bovine gamma globulin as the standard. The results shown are the mean ± SEM of two independent determinations.

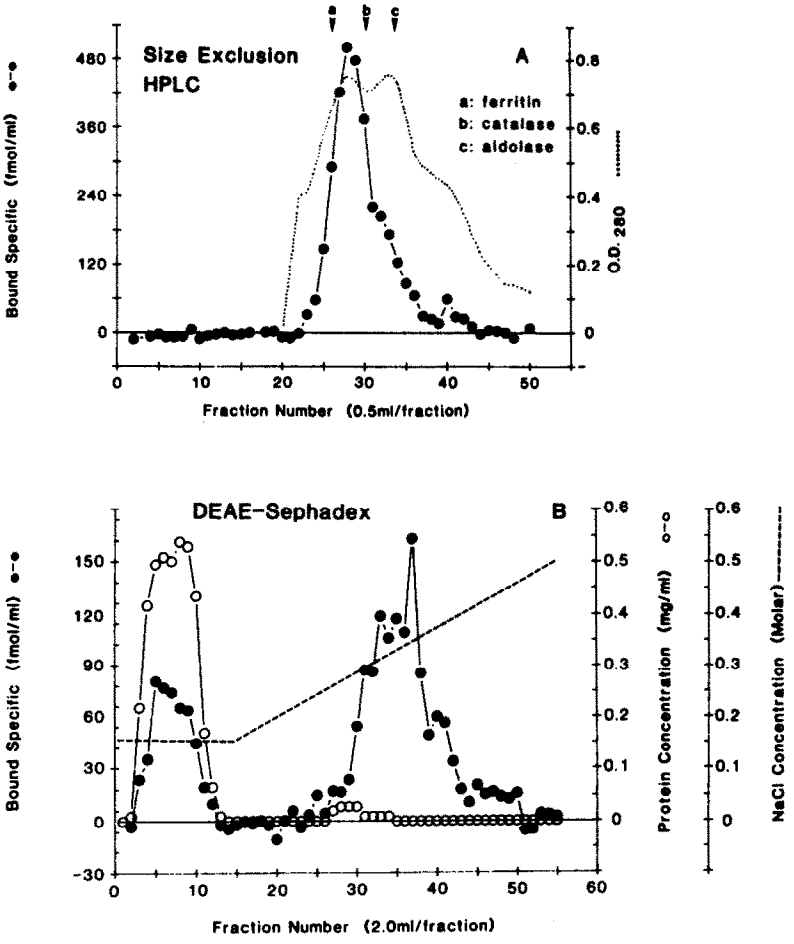

Fig. 5. Chromatographic enrichment of soluble binding sites for [^{3}H]SPD. (A) Size-exclusion HPLC was performed with digitonin-solubilized extracts of canine caudate. Five caudates were extracted with 1.0% digitonin. After centrifugation, the supernatant fraction was concentrated approximately 9-fold using an Amicon filtration system with a YM-30 filter. Aliquots (800 μl) of concentrated supernatant were applied to tandem TSK-3000 and TSK-4000 (7.5 mm × 60 cm) columns with a TSK-2-3000 precolumn (7.5 mm × 10 cm). Fractions were collected every 0.5 min. The corresponding fractions from eight chromatographic runs were pooled and assayed for the binding of [^{3}H]SPD. The binding of [^{3}H]SPD (2.0 nM) (●) was assessed using 250 μl of each fraction in a final volume of 0.5 ml using the PEG precipitation binding assay. (+)-Butaclamol (2.0 μM) was included in the assay to define the nonspecific binding of [^{3}H]SPD. A representative OD_{280} elution profile is shown. (B) Fractions from size-exclusion HPLC in which specific binding of [^{3}H]SPD was observed were pooled (fractions 25–31) and applied to a 5.0-ml DEAE-Sephadex column equilibrated with 50 mM Tris–HCl, 10 mM EDTA, 150 mM NaCl, and 0.1% digitonin at pH 7.5. After washing with 20 ml of equilibration buffer, a 150 to 500 mM linear gradient of NaCl was used to elute adsorbed protein. The binding of [^{3}H]SPD (2 nM) (●) was assessed using 0.25 ml of each fraction in a final volume of 0.3 ml with the PEG precipitation binding assay. (+)-Butaclamol (2 μM) was included in the assay to define the nonspecific binding of [^{3}H]SPD. Two-ml fractions were collected. The concentration of protein (○) in each fraction was determined by the method of Bradford [25] using bovine gamma globulin as the protein standard.

of the ligand, (2) characterize the population of soluble binding sites for [^{3}H]SPD, and (3) enrich for the solubilized binding sites using molecular-sieve and ion-exchange chromatographic techniques.

Maximal yields of solubilized binding sites for [^{3}H]SPD from canine caudate tissue homogenates were obtained with 0.75–1.0% digitonin. Higher concentrations of detergent (1.0–2.0%) increased the total amount of protein extracted, but the yield of active solubilized binding sites remained essentially constant. These results differ from those obtained in studies on the solubilization of binding sites for [^{3}H]SPD from porcine pituitary tissue [18]. In that study, a decrease in the yield of digitonin-solubilized binding sites (25–50%) was observed when tissue was extracted with increasing concentrations of digitonin (1.0–3.0%). This difference may be due simply to a variation in the amount of digitonin in the binding assay for the soluble binding sites from the pituitary. When a constant volume of detergent-solubilized extract was tested for binding activity, the final concentration of digitonin in the binding assay varied proportionally to the concentration used in the extraction. We found that, as the concentration of digitonin in the binding assay increased, the amount of specific binding of [^{3}H]SPD decreased. Therefore, in our studies, the final concentration of digitonin in the binding assays was adjusted to 0.1%. The yield of solubilized binding sites for [^{3}H]SPD from canine caudate was generally 15–18% of the binding sites present in the membranes. After extraction of caudate membranes with detergent, a portion of the binding sites remained in the pellet, but 50–60% of the sites were apparently irreversibly lost.

When agonists were used to inhibit the binding of [^{3}H]SPD to solubilized preparations, the data best fit a one-site model. This result was consistent with previously reported findings with solubilized preparations of both caudate [9, 26, 27] and pituitary [17, 18]. There was no evidence of a solubilized, high-affinity ternary complex. However, competition curves using D_2-selective antagonists were more complex than previously reported [8, 9, 26]. While the competition curves using antagonists for some preparations of solubilized canine caudate modelled as one site, most preparations possessed low-affinity sites for antagonists, which represented up to 40% of the population of solubilized binding sites for [^{3}H]SPD. Although the proportion of the binding sites with low affinity for antagonists varied from one preparation of pooled caudate to another, similar proportions of high- and low-affinity sites were found for both the soluble and membrane-associated sites in a given preparation.

Although relatively selective for the combining site of the D_2 receptor, specific binding of [^{3}H]SPD, as defined with (+)-butaclamol, can also include binding to the S_2 subtype of the serotonin receptor [28]. Previously published results of studies of the binding of [^{3}H]SPD using caudate tissue from several different species have shown that S_2 receptors contribute to the binding of [^{3}H]SPD [29–31]. Therefore, it is likely that the binding sites for [^{3}H]SPD in solubilized preparations of canine caudate with low affinity for the D_2-selective antagonists are composed primarily of S_2 receptors. Membrane homogenates and solubilized preparations have been used to compare the binding of [^{3}H]SPD in either the presence or absence of the S_2-selective antagonist ketanserin. For each preparation of pooled canine caudates, the decrease in the value of B_{max} for membrane and solubilized preparations in the presence of ketanserin was essentially identical (5–30%), indicating that the yield of solubilized D_2 and S_2 sites was the same (data not shown). In addition, the proportion of S_2 sites defined by the change in B_{max} using Scatchard analysis in the presence or absence of ketanserin was similar to the proportion of low-affinity sites for sulpiride. This result indicates that, under the conditions of our binding assay, S_2 receptors contribute to the binding of [^{3}H]SPD in both membrane and solubilized preparations of canine caudate. Our inability to observe biphasic competition curves in studies with agonists is consistent with the results of experiments carried out with membranes from frontal cortex and from caudate. Agonists are frequently less selective than antagonists, and it is difficult to use agonists to distinguish between D_2 and S_2 receptors. Extensive studies in our laboratory on membrane-associated binding sites for [^{3}H]SPD in rat caudate indicate that the binding component with low affinity for sulpiride is not composed solely of S_2 receptors. Alpha$_2$-adrenergic and spirodecanone sites also appear to contribute to the low-affinity component (P. McGonigle and P. B. Molinoff, unpublished data).

The proportion of the binding sites for [^{3}H]SPD with low affinity for antagonists has been found to vary from one preparation of pooled canine caudate to another. Although we cannot rigorously rule out the possibility that the site with a low affinity for D_2 antagonists has been generated by endogenous proteolytic enzymes, the inclusion of protease inhibitors in the buffers used for the dissection of the tissue and for radioligand binding assays did not result in monophasic competition curves for the solubilized preparations. It is also possible that the variability is due to differences in tissue dissection. Alternatively, variability in the properties of the binding sites for [^{3}H]SPD may arise due to the use of an outbred population of animals. Our own studies on the binding of [^{3}H]SPD to caudates in a variety of species (including bovine, ovine, porcine and rat) indicate that the magnitude of the component with low affinity for sulpiride varies widely, both from species to species and within a given species from one preparation to another.

Although the binding of [^{3}H]SPD to solubilized canine caudate tissue is not homogeneous, the major component responsible for the binding of [^{3}H]SPD in solubilized preparations of canine caudate appears to be the D_2 subtype of the DA receptor. This conclusion is based on the stereoselective inhibition of the binding of [^{3}H]SPD by the (+) and (−) isomers of butaclamol, preferential inhibition of binding by D_2-specific antagonists compared to S_2-selective antagonists, and the appropriate potency of agonists at DA receptors. The inhibition of the binding of [^{3}H]SPD by agonists yielded similar results to those obtained in studies of digitonin-solubilized binding sites from bovine and porcine pituitary tissue [17, 18].

Approximately 80% of the solubilized binding sites for [^{3}H]SPD adsorbed to DEAE-Sephadex in buffers containing digitonin (0.1–1.0%) and 150 mM NaCl. Binding sites could be eluted by increasing the concentration of NaCl in the buffer. However, enrichment of binding sites was accompanied by a 3- to 4-fold decrease in the apparent K_d for [^{3}H]SPD compared with the crude solubilized preparations. This change in affinity was observed although all of the binding assays contained 0.1% digitonin. The increased K_d value obtained for enriched preparations and the observed decrease in the binding of [^{3}H]SPD with increasing concentrations of digitonin suggest that the effect of increasing the detergent-to-protein ratio is to decrease the binding of the radioligand. Scatchard analysis of the binding of [^{3}H]SPD to caudate membranes and to solubilized preparations in the presence of various concentrations of digitonin indicated that increasing the concentration of detergent while maintaining a constant concentration of protein decreased both the apparent affinity and the value of B_{max} (unpublished data). However, the final concentration of digitonin in the binding assay also appears to play a role in determining how [^{3}H]SPD binds to the receptor. Scatchard analysis of the binding of the radioligand to solubilized preparations in which the detergent-to-protein ratio was kept constant, but in which the final concentration of digitonin varied, indicated that the affinity and density of binding sites appeared to decrease as the concentration of detergent in the assay increased above 0.2% (unpublished data). A similar decrease in the binding of [^{3}H]SPD in the presence of detergent was observed by Senogles *et al.* [32] when preparations of solubilized bovine pituitary, enriched for binding sites for [^{3}H]SPD using a carboxymethyloxime derivative of SPD covalently linked to epoxy-Sepharose, were compared before and after reconstitution into phospholipid vesicles.

The experiments reported in this paper provide information essential for further studies on the purification of solubilized D_2 receptors. First, solubilized caudate binding sites for [^{3}H]SPD contain a substantial proportion of sites that do not have the properties of D_2 receptors (i.e. low affinity for D_2-selective antagonists). The proportion of low-affinity sites varies from preparation to preparation of pooled canine caudate. Thus, the binding of [^{3}H]SPD is not an adequate criterion for the identification of the D_2 receptor. Second, although binding sites for [^{3}H]SPD can be solubilized with 1.0% digitonin, the interaction between ligand and binding site is optimal at lower concentrations of detergent. The concentration of digitonin in the binding assay that was found to be optimal was three to four times lower than concentrations used in most previous studies [9, 17, 18, 26, 27, 32]. A reduction in the concentration of detergent is necessary not only to maximize ligand-binding site interactions for studies on the pharmacologic properties of solubilized binding sites, but also for developing protocols for affinity chromatographic enrichment of solubilized sites using derivatives of butyrophenones. Third, enriched preparations of D_2 receptors have a lower K_d value for the binding of [^{3}H]SPD than is seen in studies with membranes. This is probably due to an increase in the detergent-to-protein ratio. Therefore, either higher concentrations of radioligand or reconstitution of solubilized receptor into lipid vesicles will be essential to assess the pharmacologic properties of purified D_2 receptors using radioligand binding techniques [5, 32]. Finally, binding sites for [^{3}H]SPD adsorb to anion exchange resins. Thus, affinity chromatography columns will be more effective if they are designed to avoid the generation of secondary and tertiary amines when ligands are covalently coupled to insoluble supports [32, 33].

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