Biochemical Pharmacology, Vol. 29, pp. 2009–2016

0006-2952/80/0715-2009 $02.00/0

SOLUBILIZATION OF MULTIPLE BINDING SITES FOR THE DOPAMINE RECEPTOR FROM CALF STRIATAL MEMBRANES*

YVONNE C. CLEMENT-CORMIER,† LAURENCE R. MEYERSON‡ and ANDREW MCISAAC†

†Departments of Pharmacology and Neurobiology, The University of Texas Medical School at Houston, Houston, TX 77025 and ‡Department of C.N.S. Research, Medical Research Division, American Cyanamid Company, Pearl River, NY 10965, U.S.A.

(*Received* 17 *November* 1979; *accepted* 23 *January* 1980)

Abstract—Specific dopamine binding sites from calf striatum were solubilized using potassium chloride. The solubilized salt extract was found to have the same properties as the native membrane preparation, including binding affinity and stereoselectivity. The binding site required the presence of potassium chloride for solubilization, but not for the maintenance of binding. Multiple binding sites were detected in the potassium chloride extract. Hill coefficients for selected agonists and antagonists using three different ligands which interact with the dopamine receptor, [^{3}H]spiroperidol, [^{3}H]-2-amino-6,7-dihydroxy 1,2,3,4-tetrahydronapthalene (ADTN) and [^{3}H]-*N*-propylnorapomorphine, revealed the presence of more than one population of agonist binding sites. In addition, gel filtration chromatography on Sephacryl S-200 demonstrated three peaks of stereospecific [^{3}H]spiroperidol binding. Similar results were obtained following gel filtration chromatography of the potassium chloride extract on Sephadex G-100. The predominant specific site which was labeled by [^{3}H]apomorphine, [^{3}H]ADTN and [^{3}H]spiroperidol on this column had a calculated molecular weight of about 50,000, as calibrated for globular proteins. These data confirm the existence of multiple binding sites for the dopamine receptor in the central nervous system.

Recently, the multiplicity and heterogeneity of the dopamine receptor have been reported by several investigators using *in vitro* binding assays. Multiple binding sites for the dopamine receptor have been identified using radiolabeled ligands such as apomorphine [1], 2-amino-6,7-dihydroxy 1,2,3,4-tetrahydronapthalene (ADTN) [2, 3], haloperidol [4, 5] and spiroperidol [6, 7]. Kinetic analysis of the binding sites using these ligands revealed biphasic saturation curves and non-linear Scatchard plots. These data, which demonstrate multiple binding sites for a given ligand [5, 8], lend support to a variety of *in vivo* evidence suggesting the existence of multiple dopamine receptors [9–12].

The ability to obtain specific [^{3}H]spiroperidol binding sites from rat and calf striatum in a soluble form [13, 14] has made it possible to isolate and study these multiple receptor sites, using established chromatographic techniques. The data which appear here present the results of such studies on the solubilization and isolation of multiple binding sites for the dopamine receptor.

EXPERIMENTAL PROCEDURE

Materials. Frozen bovine brains were obtained from Pel-Freeze and stored at −20° until use. [^{3}H]Spiroperidol (26.4 Ci/mmole), [^{3}H-*N*-propylnorapomorphine (80 Ci/mmole) and [^{3}H]ADTN (18 Ci/mmole) were purchased from New England Nuclear (Boston, MA). Unlabeled haloperidol was obtained from Janssen Pharmaceuticals (Beerse, Belgium). (+)- and (−)-Butaclamol were gifts from Ayerst Laboratories (Toronto, Ontario, Canada), and Norit SGX was purchased from the Sigma Chemical Co. (St. Louis, MO). All other drugs and chemicals were obtained from their respective commercial sources in the highest purity available.

Subcellular fractionation. Calf caudate nuclei were dissected, frozen and stored overnight at −20°. All subsequent fractionation procedures were conducted at 0–4°. The tissue was homogenized in 10 vol. of 0.32 M sucrose–2 mM Tris (hydroxymethylaminomethane maleate, pH 7.4). The crude synaptosomal fraction (P_2) and the microsomal (P_3) fractions were obtained as follows. The homogenate was centrifuged at 900 *g* for 10 min, yielding a pellet (P_1) and a supernatant fraction (S_1). The S_1 was further centrifuged at 11,500 *g* for 20 min to yield a crude synaptosomal pellet (P_2) and a supernatant fraction (S_2). The P_2 was suspended in half the original volume of the homogenizing buffer and recentrifuged for 20 min to yield a washed pellet (P_2') and supernatant fraction (S_2'). S_2 and S_2' were combined and centrifuged at 100,000 *g* for 75 min to yield a microsomal pellet (P_3) and the cytosolic fraction (S_3).

Solubilization. The P_3 microsomal pellet was resuspended in chilled 0.25 M sucrose, 15 mM Na_2HPO_4 (pH 7.2) at a volume equivalent to 20 per cent of that originally used for homogenization. KCl (50%, w/w) was added to the P_3 suspension and was immediately homogenized. Unless indicated otherwise, the salt-treated homogenate was allowed to

* This work was supported by grants from the Pharmaceutical Manufacturers Association, Merck, Sharp & Dohme, PHS-MH 32431 and BNS 7816003.

stand on ice for 15 min and was then centrifuged at 20,000 *g* to remove the excess undissolved salt. The supernatant fraction from the 20,000 *g* spin was then centrifuged at 100,000 *g* for 60 min. The resultant supernatant fraction was taken as the soluble extract.

Binding assay. The standard assay constituents for binding studies (final volume, 1.0 ml) contained 15 mM Tris–HCl (pH 7.4), 5 mM Na_4EDTA, 1.1 mM ascorbate, ^{3}H-ligand, 800 μl of the solubilized tissue preparation (400 μg protein, plus test substance as indicated. The reaction was routinely initiated by the addition of the soluble extract and was incubated for 16 hr at 0°. Similar results were obtained with incubation for 10 min at 37°. The binding reaction was terminated as described previously [14]. The method essentially involves the addition of 100 μl of a Norit SGX slurry to bind free ligand. The Norit SGX slurry contained 50 mM Tris–HCl, 8 mM theophylline, 6 mM β-mercaptoethanol containing 2% BSA, and 10% Norit SGX. The tubes were allowed to stand on ice in the presence of the slurry for 5 min and were then centrifuged for 4 min in a Brinkmann microfuge. Aliquots of the supernatant fraction (400–600 μl) were withdrawn and added to scintillation vials containing 7 ml of Biofluor (NEN). Radioactivity was measured in a Searle Mark III liquid scintillation spectrometer.

Gel filtration chromatography. Gel filtration of the soluble extract was performed on 2.4 × 50 cm columns of Sephacryl S-200 and on Sephadex G-100, equilibrated with 15 mM sodium phosphate containing 0.25 M sucrose (pH 7.0). The column jacket and effluent were maintained at 4° throughout the procedure. A 2-ml aliquot of the soluble extract (2 mg protein) was applied to the column via an automatic sample injector and was eluted at a constant pressure flow rate of 2.5 ml/min. Five-ml fractions were collected, utilizing an automatic refrigerated fraction collector (Buchler Instruments). An ultraviolet detector set at 280 nm was used to monitor the effluent peaks. Both columns were calibrated with Aldolase (158,000), BSA monomer (66,000), ovalbumin (48,000), chymotrypsinogen A (25,000), and ribonuclease A, (13,500), for molecular weight estimations.

Gel electrophoresis. The SDS system was essentially that described by Laemmli [15], with gel dimensions of 11 × 16 × 4 cm and staining for protein in 0.25% coomassie blue–10% acetic acid for 1 hr, followed by destaining overnight in 7.5% acetic acid.

Protein determination. Protein concentrations were determined by the method of Lowry *et al.* [16] or Bradford [17].

RESULTS

[^{3}H]Spiroperidol binding sites from calf striatum were solubilized using salt extraction with KCl. The results in Table 1 show the effect of various solubilizing agents on the extraction of a macromolecular component from the striatum which exhibits specific [^{3}H]spiroperidol binding. Detergents such as Triton and Lubrol WX did not produce a significant increase in specific binding activity, as defined by displacement in the presence of 10^{-7} M (+)-butaclamol. The largest number of binding sites was observed when KCl was used as the solubilizing agent. The recovery of the soluble receptor, compared to the membrane bound site, averaged between 20 and 30 per cent. Specific [^{3}H]spiroperidol binding in the KCl-treated extract represented a 2-fold increase in binding over that observed in the crude striatal homogenate. Concentrations of detergent greater than 1%, up to 10%, did not alter the number of specific [^{3}H]spiroperidol binding sites (data not shown) from that reported in Table 1. These data do not rule out the possibility that variations in the concentration of detergent as well as other biochemical variables such as pH, ionic strength and stabilizing agents, may yield a preparation exhibiting specific binding activity. In addition, the use of frozen tissue, which seems to be required for KCl extraction, is probably not optimal for detergent extraction, since most procedures using detergents utilize fresh tissue.

Table 1. Effect of various solubilizing agents on [^{3}H]spiroperidol binding*

Test substance	Specific [^{3}H]spiroperidol binding (fmoles/mg protein)
KCl	11.0
Deoxycholate	0
Octyl-8-D-glucopyranoside	0
Brij-35	0.4
Brij-W-1	0.8
Triton X-100	0.6
Triton X-207	0
Triton X-305	1.4
Triton X-405	0.8
Triton X-15	0
Triton CF-21	0.2
Triton B-1956	0.4
Triton QS-15	0
Lubrol WX	0
Lubrol DX	0
NP 40	0.1
Digitonin	0.4

* The data are taken from a representative experiment testing the effects of various solubilizing agents on a microsomal pellet of the calf striatum. All agents were suspended in 15 mM K_2HPO_4, pH 7.4, at the indicated concentrations. The error range for the values shown is approximately 10 per cent from samples assayed in triplicate. The concentration of all detergents used was 0.1% except for digitonin which was 0.2%. The concentration of KCl was 400% (w/w). The concentration of [^{3}H]spiroperidol was 0.2 nM.

The displacement of [^{3}H]spiroperidol binding to the sites in the KCl-P_3 extract by various neuroleptic drugs is shown in Table 2. The concentration of (+)-butaclamol which displaced 50 per cent of the total counts (5×10^{-9} M) was equal to that of the particulate site. Other neuroleptic drugs and agonists of dopamine had IC_{50} values for [^{3}H]spiroperidol binding in the soluble fraction that were equivalent to those observed in the particulate fraction. Removal of the KCl present in the extract by overnight dialysis neither altered the binding activity for