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PURIFICATION OF THE D-2 DOPAMINE RECEPTOR FROM BOVINE STRIATUM

Zvulun Elazar, Hannah Kanety, Carol David and Sara Fuchs

Department of Chemical Immunology
The Weizmann Institute of Science
Rehovot, 76100, Israel

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The D-2 dopamine receptor has been purified 21500 fold from bovine striatal membranes. Solubilized receptor preparation was partially purified by affinity chromatography on a haloperidol adsorbent followed by gel filtration on a Sephacryl S-300 column. The fractions eluted from this column which contained the ligand binding activity were further chromatographed on wheat germ agglutinin conjugated to Sepharose. The resulting receptor preparation displays a major polypeptide band of an apparent molecular weight of 92 kDa, and exhibits a specific binding activity of 2490 pmol spiperone per mg protein. This purified receptor preparation can readsorb specifically to the haloperidol affinity column indicating that the 92 kDa polypeptide represents the ligand binding unit of the D-2 dopamine receptor.

The D-2 dopamine receptor has recently been solubilized from membranes of pituitary gland and striatum by use of various detergents and its pharmacology is well defined (reviewed in 1,2). This receptor is a glycoprotein as indicated by its specific adsorption to and elution from wheat germ agglutinin-Sepharose (3). In order to elucidate the molecular mechanisms by which dopaminergic signal transduction correlates with dopaminergic functions, it is essential to isolate and fully characterize this receptor. Purification of the D-2 dopamine receptor has been impeded both by the minute quantities available and by the lack of specific ligands suitable for affinity purification. Affinity resins based on several dopaminergic antagonists of the butyrophenone family have been prepared recently and employed for the isolation of ligand-specific antibodies and of the dopamine receptor itself (4-7). Photoaffinity labeling of the D-2 dopamine receptor has indicated that, in a variety of mammalian tissues and species, a 92-94 kDa polypeptide is probably the ligand binding unit of the receptor (8-11). In this report we describe a

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purification procedure of the D-2 dopamine receptor yielding a preparation which exhibits a single major band on SDS-polyacrylamide gel electrophoresis, with an apparent molecular weight of 92 kDa and a specific binding activity of 2490 pmol spiperone per mg protein.

MATERIALS AND METHODS

Materials: [^{3}H]-spiperone (24.8 Ci/mmol) and [^{3}H]-haloperidol (18.7 Ci/mmol) were purchased from New England Nuclear (Boston). Haloperidol and spiperone were gifts from Janssen Pharmaceuticals (Beerse). Butaclamol [(+) and (-)] and mianserine were obtained from Research Biochemicals Inc. (Wayland, MA). 3-[(3-cholamidopropyl) -dimethylammonio]-1 propanesulfonate (CHAPS), N-acetylglucosamine, phenylmethylsulfonyl fluoride (PMSF) and leupeptin were obtained from Sigma. Sepharose-wheat germ agglutinin (Sepharose-WGA), Sephacryl S-300 and molecular weight standards were purchased from Pharmacia.

Membrane preparation and solubilization: Bovine striatal membranes were prepared and solubilized essentially as described previously (12), with the addition of leupeptin (20 μg/ml) in the solubilization buffer.

Purification procedure: Affi-gel 10 (Bio-Rad) conjugated with haloperidol glycine ester (HGE) was prepared as described previously (13). Before use, the gel was washed with deionized water and mixed with an equal volume of Sepharose 4B. This adsorbent, designated affi-gel-HGE, was washed with 10 bed volumes of a buffer containing 50 mM HEPES pH 7.4, 5 mM $MgCl_2$ and 1 mM EDTA (HME buffer), supplemented with 1 mM CHAPS, 150 mM NaCl, 1 mM DTT and 1 mM PMSF (wash buffer). The washed adsorbent was mixed with 5 volumes of the solubilized membrane preparation that had been diluted 1:1 with HME buffer, and the mixture was incubated with gentle shaking for 16 hr at 4°C, or for 1 h at 22°C. The supernatant was removed and the gel was washed three times, each with 5 bed volumes of wash buffer. Elution was carried out with 10 μM spiperone in wash buffer for 40 min at 22°C, and the eluted solution was concentrated by centricon 10 (Amicon) and designated eluate I.

Eluate I (0.5 ml, 0.1 mg protein/ml) was chromatographed on a Sephacryl S-300 column (0.5 x 60 cm) and eluted with wash buffer at a flow rate of 4 ml/h. Fractions of 1.4 ml were collected and samples were analyzed by SDS polyacrylamide gel (7-15%) electrophoresis (SDS-PAGE) (14) or by [^{3}H]-spiperone binding. For the SDS-PAGE analysis, an aliquot of eluate I was radioiodinated (15) and mixed with unlabeled preparation prior to chromatography on Sephacryl S-300. Following gel filtration, the fractions containing the [^{3}H]-spiperone binding activity were pooled and added to Sepharose-WGA in a ratio of 7/1 (v/v). The adsorbed material was eluted with 0.2M N-acetylglucosamine in wash buffer. The eluted material (eluate II) was concentrated and assayed for [^{3}H]-spiperone binding or by SDS-PAGE.

Reconstitution of D-2 receptor activity: Reconstitution of D-2 receptor activity was performed as described by Cerione et al. (16) for the β-adrenergic receptor, with some modifications. The tested samples (50-200 μl) were incubated with HME buffer containing 100 mM NaCl, bovine serum albumin (2 mg/ml), sonicated phosphatidylcholine (0.7 mM) and octyl-β-D- glucoside (0.8%), in a final volume of 0.5 ml for 15 min at 4°C and then dialyzed against 1000 volumes of HME buffer containing 1 mM PMSF, 100 mM NaCl and Bio-Beads SM-2 resin (2 g/l) for 16 hr at 4°C.

Binding assays: Specific binding of [^{3}H]-spiperone and [^{3}H]-haloperidol to receptor preparations was determined as described previously (12).

Protein determination: Protein concentrations were determined by Bradford micro assay (17), fluorescamine assay (18), and/or trace radioiodination.

RESULTS

The first step of purification of the D-2 dopamine receptor was affinity chromatography on an adsorbent of haloperidol glycine ester coupled to affi-gel 10 (affi-gel-HGE). This adsorbent was found to adsorb 70-80% of the [^{3}H]-spiperone binding activity and less than 5% of the total proteins from a CHAPS solubilized membrane preparation. Adsorption of the [^{3}H]-spiperone binding activity was inhibited by preincubation of the solubilized preparation with spiperone or (+)-butaclamol and to a lesser extent by haloperidol, apomorphine or dopamine. (-)-Butaclamol and the serotonergic antagonist mianserine had no significant effect (Fig. 1). This profile demonstrates that the affinity matrix displays the predicted specificity for the D-2 dopamine receptor. Approximately, 0.1% of the total protein applied to the affi-gel-HGE matrix was eluted with 10 μM spiperone (Table I). Elution with spiperone was more efficient than that obtained with other dopaminergic antagonists such as

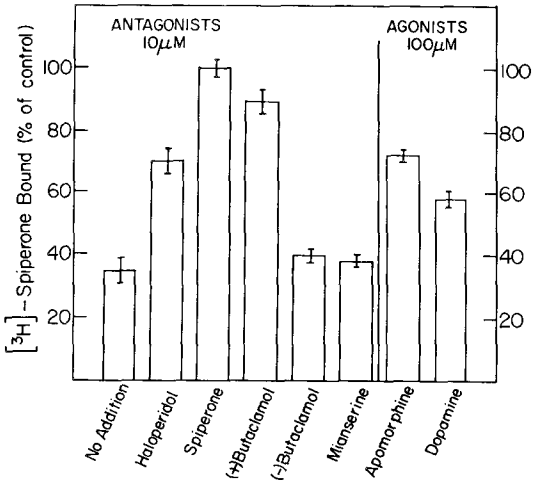

Fig. 1: Specificity of adsorption of solubilized D-2 dopamine receptor to affi-gel-HGE. Two milliliters of solubilized receptor preparation were preincubated with the indicated ligands for 40 min at 22°C and applied to 0.4 ml of affi-gel-HGE for 1 h at 22°C. The effluent was desalted on Sephadex G-50 mini columns and assayed for [^{3}H]-spiperone binding. The results are expressed as the percent of a control in which the solubilized receptor was incubated with affi-gel-ethanolamine. Parallel experiments were also performed to correct for the incomplete removal of the ligands by desalting.

TABLE I: Purification of D-2 dopamine receptor from bovine striatum

Step	Volume (ml)	Protein conc. (mg/ml)	Specific activity (pmole[^{3}H]-spiperone/ mg protein)	Purification (fold)	Recovery (%)
Membranes	25	10	0.116	1	100
CHAPS extract	25	2.45	0.20	1.72	42
Affi-gel-HGE (eluate I)	5	0.01	96.7[a]	833	17
Sephacryl	4.2	0.002	330[a]	2840	9.6
Sepharose-WGA (eluate II)	0.5	<0.0002	2490[a]	21460	0.9

[a] [^{3}H]-spiperone binding was measured following reconstitution into phosphatidylcholine liposomes.

haloperidol and (+)-butaclamol (data not shown). The eluted material (eluate I) had to be reconstituted by insertion into artificial phosphatidylcholine liposomes before assaying for [^{3}H]-spiperone binding activity. This step resulted in a 500 fold purification, and about 40% of the binding activity applied to the affi-gel-HGE was recovered (Table I).

Eluate I retained the dopaminergic antagonist specificity and stereospecificity (Fig. 2). Spiperone and (+)-butaclamol inhibited the [^{3}H]-spiperone binding to eluate I with IC_{50} values of 0.8 nM and 1.6 nM, respectively, while the IC_{50} of (-)-butaclamol was at least 3 orders of magnitude higher. The affinities of [^{3}H]-haloperidol and [^{3}H]-spiperone (K_D values of 1.32 $\pm$ 0.29 and 0.63 $\pm$ 0.17 nM, respectively) for eluate I were similar to their affinities (1.26 $\pm$ 0.25 and 0.52 $\pm$ 0.15 nM, respectively) for the solubilized preparation.

Radioiodinated eluate I exhibited several protein bands on SDS-PAGE (Fig. 3A, lane 1). To elucidate which protein(s) corresponds to the D-2 dopamine receptor, the solubilized receptor preparation was preincubated with spiperone (10 μM) prior to application on affi-gel-HGE, and subsequently eluted with spiperone. These conditions, in which the D-2 dopamine receptor does not bind

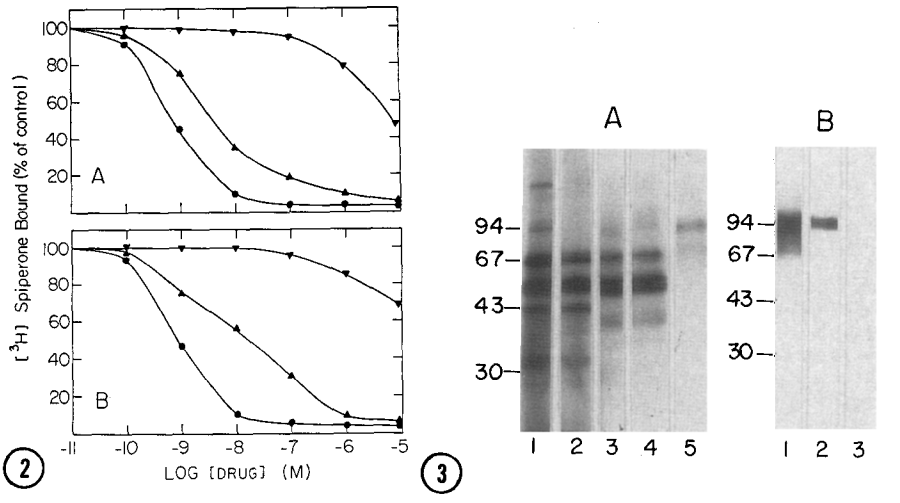

Fig. 2: Binding specificity of reconstituted affinity-purified receptor preparation. Solubilized receptor (A) or affinity purified-reconstituted receptor (B) were incubated in HME buffer with [^{3}H]-spiperone (3-5 nM) and increasing concentrations of spiperone (●), (+)-butaclamol (▲) and (-)-butaclamol (▼). The 100% [^{3}H]-spiperone bound corresponds to 160-180 pM (in A) and 150-170 pM (in B).

Fig. 3: A). SDS-PAGE analysis of radioiodinated D-2 receptor preparations. 1, eluate I; 2, eluate obtained when the solubilized receptor preparation was preincubated with 10 μM spiperone prior to chromatography on affi-gel-HGE; 3, pooled fractions 23-25 from the Sephacryl-300 column; 4, unbound material following adsorption of fractions 23-25 on Sepharose-WGA; 5, eluate II.
B). Readsorption of eluate II on affi-gel-HGE. Radiolabeled eluate II (1) was applied to affi-gel-HGE (2) or to affi-gel-ethanolamine (3) and eluted with Laemmli sample buffer.

to affi-gel-HGE (Fig. 1), resulted in a depletion of protein bands of 92 kDa and 140 kDa, and also in a decrease in the amount of additional bands of 30 and 14 kDa (Fig. 3A, lane 2).

The affinity purified receptor preparation (eluate I) was further chromatographed on a Sephacryl S-300 column, resulting in an additional enrichment of the receptor binding activity (Table I). The fractions eluted from this column were analyzed by ligand binding activity or by SDS-PAGE (Fig. 4). The fractions which included the binding activity were devoid of several major proteins present in eluate I and contained the aforementioned 92 kDa band. Fractions 23-25, which contained the ligand binding activity, were pooled, concentrated and loaded onto Sepharose-WGA. Most of the proteins were not adsorbed to the Sepharose-WGA resin, whereas the 92 kDa polypeptide was adsorbed and specifically eluted with N-acetylglucosamine (Fig. 3A, lanes

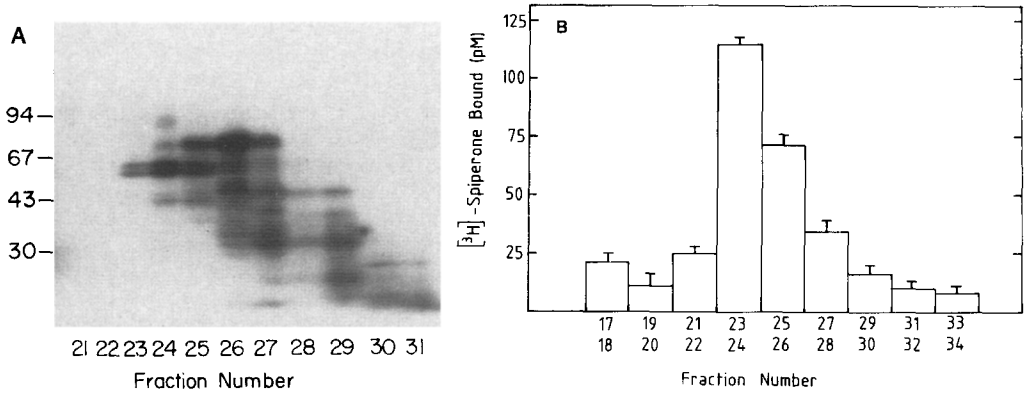

Fig. 4: Gel filtration of eluate I. A). Radiolabeled eluate I was mixed with the same volume of concentrated unlabeled eluate I and chromatographed on a Sephacryl S-300 column. Fractions were concentrated and electrophoresed on a 7-15% SDS-polyacrylamide gel. B). Unlabeled eluate I was chromatographed on Sephacryl S-300. Fractions were pooled, concentrated, reconstituted and [^{3}H]-spiperone binding was determined.

3-5). When radioiodinated, this purified preparation (eluate II) exhibited a single band on SDS-PAGE. When assayed for [^{3}H]-spiperone binding activity, following reconstitution into artificial vesicles, eluate II bound 2490 pmoles spiperone per mg protein (Table I). This binding was specifically inhibited by excess of either spiperone, haloperidol or (+)-butaclamol. In addition, samples of radiolabeled eluate II were shown to specifically readsorb to affi-gel-HGE (Fig. 3B). This further identifies the 92 kDa protein as the D-2 dopamine receptor binding unit.

DISCUSSION

This paper describes a purification procedure for the D-2 dopamine receptor from bovine striatum. The affinity chromatography step on affi-gel-HGE led to partial purification of the receptor. Mainly the 92, and occasionally 140 kDa protein bands were specifically depleted from the eluate by preincubation with spiperone. The nature of the 140 kDa polypeptide is unknown. It might represent a complex of the 92 kDa protein with another polypeptide or a precursor of the D-2 receptor binding unit. Some of the other proteins which elute with spiperone might represent functional components, e.g. G-proteins, which may be specifically coupled to the receptor complex. Indeed, preliminary experi-

ments indicate that eluate I contains G_i and G_o proteins that are functionally associated with the D-2 dopamine receptor (19).

Several lines of evidence provided in this report indicate that the purified 92 kDa polypeptide is the D-2 receptor binding unit. Firstly, the peak of [^{3}H]-spiperone binding activity eluted from the Sephacryl column correlates with the presence of the 92 kDa polypeptide (Fig. 4) and, of the proteins present in this peak, only the 92 kDa band could readsorb to the haloperidol affinity resin (unpublished data). Secondly, the specific binding activity in eluate II, is 20-30% of the theoretical value, assuming one ligand binding site per receptor molecule. Taking into account some inactivation during the purification and reconstitution, and possible incorrect insertion of the receptor into artificial liposomes, this specific activity is consistent with the expected value. Finally, the purified 92 kDa protein from Sepharose-WGA can specifically readsorb to the affi-gel-HGE matrix (Fig. 3B).

The combination of ligand affinity chromatography together with gel filtration and chromatography on Sepharose-WGA provides an improved procedure for purification of the D-2 dopamine receptor. We are now attempting to improve the yields in the various purification steps so as to obtain sufficient amounts of purified D-2 receptor for the production of specific antibodies, as well as for structural analysis of the receptor.

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