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Purification of D_2 dopamine receptor by photoaffinity labelling, high-performance liquid chromatography and preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis

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ABSTRACT

[^{125}I]N-azidophenethylspiperone ([^{125}I]azido-NAPS) was used as a photoaffinity ligand for bovine D_2 dopamine receptor. On photolysis, [^{125}I]azido-NAPS was covalently incorporated into a major band of 94 kDa in bovine striatal membrane as assessed by autoradiography after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide gel). The labelled D_2 receptor protein from striatal membrane was solubilized and subjected to HPLC using gel filtration (TSK G3000SW) and hydroxyapatite gel (Pentax SH2010C), followed by two steps of preparative SDS-PAGE. The D_2 receptor protein could be obtained as a single major polypeptide on SDS-PAGE by either silver staining or autoradiography.

INTRODUCTION

Dopamine is a typical neurotransmitter in the central nervous system. Brain dopamine receptor has been classified into two groups, D_1 and D_2, based on their affinity toward dopaminergic ligands and the relationship to adenylate cyclase. D_1 receptor is linked to adenylate cyclase, whereas D_2 receptor is either negatively or not directly linked to this enzyme, but has not yet been fully characterized. D_2 dopamine receptor is of particular interest because it is associated with certain clinical conditions, *e.g.*, schizophrenia and Parkinson's disease, or their drug treatment[1,2]. Purification of this receptor has remained a formidable task owing to the minute amounts available

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and the instability of ligand binding activity. Recently, several papers have reported the purification of D_2 dopamine receptor[3–8]. However, few studies have succeeded in showing this receptor protein as an apparent single polypeptide on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

A high-affinity radioiodinated probe of D_2 dopamine receptor, [^{125}I]N-azidophenethylspiperone ([^{125}I]azido-NAPS), has been synthesized and used for the characterization of D_2 dopamine receptor[9–12]. We photolabelled the D_2 dopamine receptor of bovine striatal membrane with this ligand, and found that the labelled D_2 dopamine receptor protein showed an unusual mobility on SDS-PAGE. We utilized this characteristic for the purification of this protein. The D_2 dopamine receptor protein was purified to an apparent homogeneous polypeptide by high-performance liquid chromatography (HPLC) and preparative SDS-PAGE, using photoaffinity-labelled receptor protein as a tracer.

This method, described here, was simple and efficient in comparison with the other purification procedures reported previously.

EXPERIMENTAL

Chemicals and reagents

[^{125}I]Azido-NAPS (2200 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.) and [^{3}H]spiperone (85 Ci/mmol) from Amersham (Amersham, U.K.). The following drugs were obtained from Research Biochemical (Natick, MA, U.S.A.): (+)- and (−)-butaclamol, haloperidol, sulpiride, mianserin, propranolol and SCH23390. E-64 was obtained from Taisho Pharmaceutical (Tokyo, Japan). Leupeptin and phenylmethylsulphonyl fluoride (PMSF) were purchased from Boehringer (Mannheim, F.R.G.). All other chemicals were of the highest available purity.

Preparation of bovine synaptic membranes

Fresh bovine brains were obtained from a local slaughterhouse. Bovine striata were dissected out, weighted and frozen at −80°C until used. All subsequent steps were performed at 0–4°C. The striata were minced and homogenized with a Waring blender followed by a Potter–Elvehjem homogenizer in buffer A [0.25 *M* sucrose–10 m*M* Tris-HCl (pH 7.4)–2 m*M* EDTA–0.5 m*M* PMSF–1 μg/ml pepstatin–1 μg/ml leupeptin–1 μg/ml E-64]. The homogenate was centrifuged at 870 *g* for 10 min and the supernatant was recentrifuged at 17 600 *g* for 35 min. The pellet was then suspended in buffer B [10 m*M* Tris–HCl (pH 7.4)–2 m*M* EDTA–0.5 m*M* PMSF–1 μg/ml pepstatin–1 μg/ml leupeptin–1 μg/ml E-64] for osmotic shock, followed by centrifugation at 17 600 *g* for 35 min. The resulting pellet was resuspended and homogenized in buffer B, then the sucrose concentration of this preparation was adjusted to 0.8 *M*. The sample was layered over 1.3 *M* sucrose and centrifuged at 70 000 *g* for 2 h. The resulting band above the 1.3 *M* sucrose phase was collected, homogenized and stored at −80°C until further use.

Photoaffinity labelling

The stored bovine synaptic preparation was washed and suspended in buffer C [50 m*M* Tris–HCl (pH 7.4)–100 m*M* NaCl–2 m*M* $MgCl_2$–0.5 m*M* PMSF–1 μg/ml pepstatin–1 μg/ml leupeptin–1 μg/ml E-64]. This preparation (2 mg of protein) was

incubated (in the dark) with [^{125}I]azido-NAPS (*ca.* 30 pmol) for 2 h at 37°C in a final volume of 1 ml and processed for photolysis. Samples were irradiated for 5 min with a Hitachi 700-W mercury lamp at a distance of 20 cm, transferred to a 1.5-ml Eppendorf microfuge tube and sedimented at 17 500 *g* for 30 min. The resulting pellet was stored at −80°C. For analysis of the labelled band, samples were dissolved in sample buffer containing 10% SDS and 1% β-mercaptoethanol (β-ME) with gentle stirring for 1–2 h at room temperature and electrophoresed[13]. After the gel had dried, autoradiography was performed with intensifying screens for 1–2 days at −80°C. For large-scale preparation, we used 10-fold larger volumes than those described above.

Gel permeation chromatography

The membrane preparation labelled with [^{125}I]azido-NAPS was mixed with unlabelled preparation and solubilized with 10 m*M* phosphate buffer (pH 6.6) containing 10% SDS and 1% β-ME. The solubilized sample was chromatographed on a TSK G3000SW molecular exclusion column (60 cm × 7.5 mm I.D.) (Tosoh, Tokyo, Japan) at a flow-rate of 3 ml/min at 22°C using a Hitachi Model L6000 HPLC pump and a model L4000 UV detector. The mobile phase was 10 m*M* phosphate buffer (pH 6.6)–3% SDS. The continuous protein profile of the column eluate was monitored by measuring the absorbance at 280 nm. The column eluate was fractionated (3 ml each) and the radioactivity of each fraction was determined with a γ-scintillation counter.

Hydroxyapatite chromatography

A Pentax SH2010C HPLC column (10 cm × 21.5 mm I.D.) (Asahi Optical Industry, Tokyo, Japan) was used for hydroxyapatite chromatography. After gel permeation chromatography, the fractions containing photolabelled receptor protein were collected and diluted with two volumes of 10 m*M* phosphate buffer (pH 6.6). The sample was loaded on a hydroxyapatite HPLC column at flow-rate of 2.5 ml/min at 22°C. The mobile phase was a 10–500 m*M* linear gradient of phosphate buffer (pH 6.6)–1% SDS. The absorbance of the column eluate was monitored at 280 nm. The column eluate was fractionated (2.5 ml) and the radioactivity of each fraction was determined with a γ-scintillation counter.

SDS-PAGE

SDS-PAGE was carried out according to the procedure described by Laemmli[13]. For analytical SDS-PAGE, a 1.0-mm thick slab gel was used, whereas a 3.0-mm thick slab gel was used for preparative SDS-PAGE. Sample preparation for preparative SDS-PAGE was carried out as follows: the fractions containing photolabelled receptor protein in the hydroxyapatite HPLC eluate were collected, dialysed against 25 m*M* Tris–HCl buffer (pH 6.8) containing 0.1% SDS and concentrated using a CentriCell centrifuge ultrafilter (Polysciences, Warrington, PA, U.S.A.). The buffer composition of this sample was adjusted to that of the SDS-PAGE sample buffer [62.5 m*M* Tris–HCl (pH 6.8)–1% SDS–10% glycerol–1% β-ME] and then applied to a 3.0-mm thick slab gel containing 4% acrylamide stacking gel and 6% acrylamide separation gel. Electrophoresis was carried out overnight with electrophoresis buffer [25 m*M* Tris–HCl (pH 8.9)–192 m*M* glycine–0.1% SDS]. After the molecular marker had been stained with Coomassie Brilliant Blue R-250 (CBB), the gel containing photolabelled protein was cut off at the proper position assessed with a standard

molecular marker. The gel was then loaded directly on the stacking gel (4% acrylamide) and the second SDS-PAGE (12% acrylamide preparation gel) was performed. After electrophoresis, the receptor protein was electroeluted from the 12% acrylamide gel using Biotrap BT-1000 (Schleicher & Schüll, Keene, NH, U.S.A.). The resulting sample was stored at −80°C.

Binding assay

Binding assays of bovine synaptic membranes were carried out according to the procedure described by Ramwani and Mishra[3] with slight modification. The total binding of [^{3}H]spiperone to the preparation was determined in a 1.0-ml assay volume containing 1 n*M* [^{3}H]spiperone, whereas non-specific binding was determined in a parallel assay in the presence of 1 μM (+)-butaclamol. Specific binding is defined as the difference in counts detected in the absence and presence of 1 μM (+)-butaclamol.

Protein determination

Proteins were determined by the method of Lowry *et al.*[14] with bovine serum albumin as a standard.

RESULTS

Preparation of bovine synaptic membrane

Bovine synaptic membrane was prepared from 150 g of bovine striata (fifteen cows). The amount of the total membrane protein was 750 mg–1.0 g when it was determined by the Lowry *et al.* method. When a ligand binding assay was performed, the membrane preparation exhibited a maximum specific binding (B_{max}) of 460 fmol/mg and an equilibrium dissociation constant (K_D) of 0.26 n*M* by Scatchard analysis (data not shown).

Photoaffinity labelling

The bovine synaptic membrane preparation was photoaffinity labelled with [^{125}I]azido-NAPS, electrophoresed and rendered visible by autoradiography. [^{125}I]-Azido-NAPS was incorporated into a major polypeptide of $M_r \approx 94\,000$ (10% acrylamide gel). This photoaffinity labelling was blocked by dopaminergic ligands such as (+)-butaclamol, haloperidol and sulpiride, whereas it was not blocked by mianserin (serotonin S_2 receptor antagonist), propanolol (adrenergic receptor antagonist) and SCH23390 (dopamine D_1 receptor specific antagonist) (Fig. 1). Therefore, the broad band of $M_r \approx 94\,000$ represents the D_2 dopamine receptor protein; this finding coincides with those in other reports[9,10,12]. We measured the radioactivity of the acrylamide gel containing labelled polypeptide of $M_r \approx 94\,000$, and calculated the uptake rate of [^{125}I]azido-NAPS from the specific radioactivity of this ligand and a maximum specific binding (B_{max}) of the membrane preparation. We estimated that [^{125}I]azido-NAPS incorporated to about 2% of D_2 dopamine receptor protein in the synaptic membrane preparation.

We found that the labelled polypeptide of $M_r \approx 94\,000$ has an unusual mobility on SDS-PAGE, *viz.*, the mobility of this polypeptide relative to that of the molecular marker protein varied with the acrylamide concentration of the separation gel. The apparent molecular weight of this polypeptide in each acrylamide concentration was

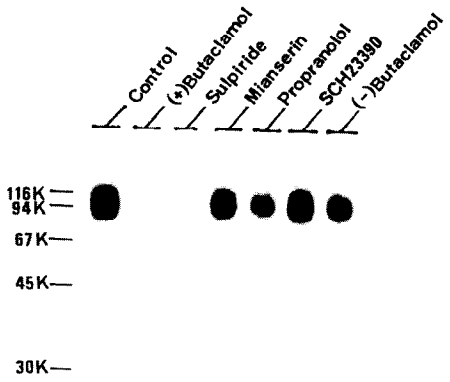

Fig. 1. Selectivity and specificity of the photoaffinity labelling of bovine striatal membrane with [^{125}I]azido-NAPS. The membrane preparation was photoaffinity labelled with [^{125}I]azido-NAPS alone (control) or in the presence of the indicated competing ligand (100 n*M*). Samples were then solubilized, electrophoresed on 10% acrylamide gel and rendered visible by autoradiography.

estimated roughly as 78 000 (6%), 88 000 (7.5%), 94 000 (10%) and 130 000 (12%) from the electrophoretic mobility of the middle portion of the broad band (Fig. 2). When CBB staining was performed, the other proteins in the membrane preparation were stained in a constant position, even if the acrylamide concentration was changed.

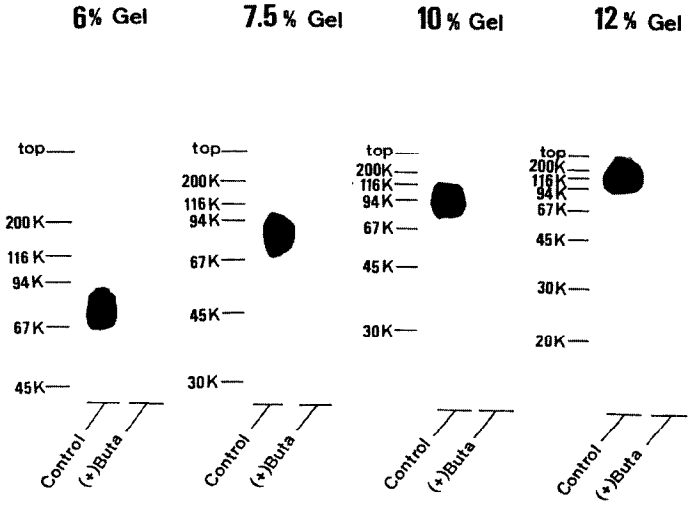

Fig. 2. SDS-PAGE pattern of the labelling with [^{125}I]azido-NAPS at several acrylamide concentrations. Membranes were photolabelled with [^{125}I]azido-NAPS alone (control) and in the presence of 100 n*M* (+)-butaclamol [(+)Buta]. Samples were then electrophoresed on different acrylamide gels as indicated, and rendered visible by autoradiography. Each gel contains the 4% acrylamide stacking gel and different acrylamide (6%, 7.5%, 10% or 12%) preparation gels.

Therefore, the shift of the mobility on SDS-PAGE seemed to be characteristic for D_2 dopamine receptor protein.

Gel permeation chromatography

The photoaffinity-labelled membrane preparation was solubilized and chromatographed on size-exclusion HPLC columns as described under Experimental. The elution profile of the protein and specific labelling in gel permeation HPLC is shown in Fig. 3a. Specific binding was determined by subtracting the counts in the eluate prelabelled in the absence and presence of 1 μM (+)-butaclamol in a parallel

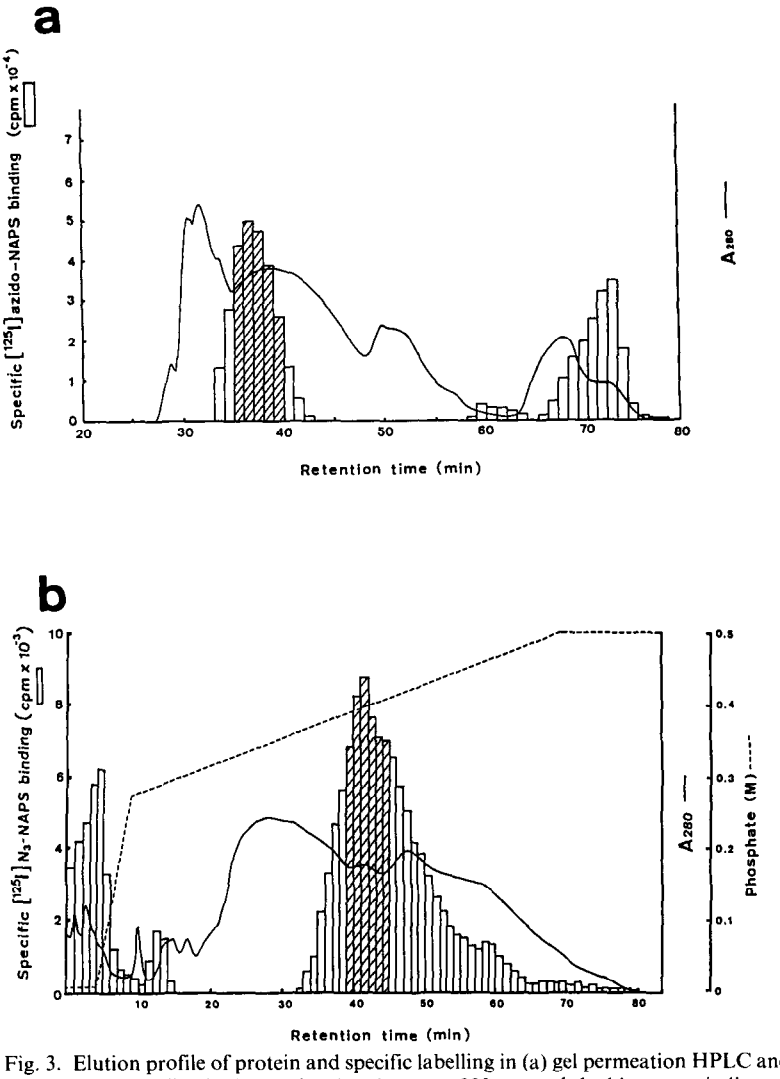

Fig. 3. Elution profile of protein and specific labelling in (a) gel permeation HPLC and (b) hydroxyapatite HPLC. The solid line indicates the absorbance at 280 nm and the histogram indicates the radioactivity of each fraction. The fractions indicated on the histogram with an oblique line were applied to the next step.

experiment. The peak of specifically labelled receptor protein with [^{125}I]azido-NAPS consistently eluted at a retention time of 37.0 $\pm$ 4.0 min (n = 40). We analysed this fraction and detected the labelled polypeptide of $M_r \approx 94\,000$ by SDS-PAGE (10% acrylamide gel), followed by autoradiography. On the other hand, the labelled polypeptide was not detected in the minor radioactive peaks eluted later. These peaks may contain the free radioactive ligand and degraded low-molecular-weight substances. Fractions 35–39, which actually contains the D_2 dopamine receptor protein, were collected and applied to the next step. The recovery of membrane protein was determined by the Lowry *et al.* method, and the recovery of D_2 dopamine receptor protein was assessed by counting the radioactivity of [^{125}I]azido-NAPS using a γ-scintillation counter. The recovery of the protein and the ligand binding activity in this step were about 20% and 80%, respectively, resulting in about a 4-fold purification.

Hydroxyapatite chromatography

In Pentax SH2010C hydroxyapatite chromatography, the labelled protein with [^{125}I]azido-NAPS was eluted as a broad peak in about 0.4 M phosphate buffer (Fig. 3b). Specific binding was determined by the difference in a parallel experiment in the absence and presence of (+)-butaclamol, and confirmed by autoradiography after SDS-PAGE. Fractions 39–44, which contained the D_2 receptor protein, were collected, dialysed and concentrated using the CentriCell. The sample was then applied to the two steps of preparative SDS-PAGE. The recovery of the protein and ligand binding activity in the hydroxyapatite chromatography step were 11% and 31%,

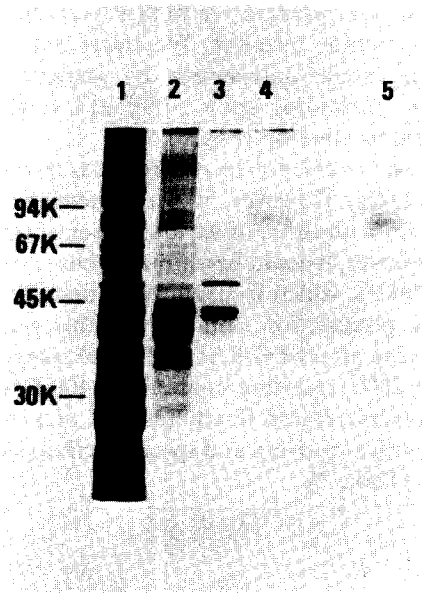

Fig. 4. SDS-PAGE pattern of the preparation of each purification step. Lanes 1–4 were made visible by silver staining and lane 5 by autoradiography. Lane 1, solubilization of bovine synaptic membrane; 2, gel permeation HPLC; 3, hydroxyapatite HPLC; 4 and 5, two steps of preparative SDS-PAGE.

TABLE I

PURIFICATION OF D_2 DOPAMINE RECEPTOR

Preparation	*Specific [^{3}H]spiperone binding*				
	Binding (cpm) (A)	*Protein (mg) (B)*	*(A)/(B) (cpm/mg)*	*Purification (-fold)*	*Yield (%)*
SDS solubilized	264 900	100	2650	1	100
Gel filtration HPLC eluate	204 200	22	9280	3.5	77
Hydroxyapatite HPLC eluate	63 400	2.5	25 360	9.6	24
Preparative SDS-PAGE eluate	23 000	0.0007	$3.54 \cdot 10^7$	13 400	8.7

respectively, when they were determined by the same methods as those in gel permeation chromatography. This step resulted in about a 3-fold purification.

Preparative SDS-PAGE

The partially purified sample obtained by gel permeation chromatography and hydroxyapatite chromatography was applied to the two steps of preparative SDS-PAGE. After electrophoresis on 6% acrylamide preparation gel, the gel was cut off at the position of M_r 60 000–90 000, which contained photolabelled protein. This gel was loaded on 12% acrylamide preparation gel, electrophoresed and then gel was cut off at the position above M_r 94 000. The labelled protein was electroeluted by Biotrap BT-1000 with high efficiency. This sequential preparative SDS-PAGE step was very effective for the purification of D_2 dopamine receptor. We could obtain D_2 dopamine receptor protein as an apparent single polypeptide by analytical SDS-PAGE followed by silver staining. Further, autoradiography exhibited a single band at the same position as that detected by silver staining (Fig. 4).

The recovery of receptor protein in this step could not be determined by the Lowry *et al.* method, because it was undetectable. However, it was determined approximately from the staining density compared with those of various doses of the standard marker proteins after SDS-PAGE. Moreover, it could be estimated based on the recovery of the radioactivity of [^{125}I]azido-NAPS, which incorporated about 2% of D_2 dopamine receptor protein, because the preparation obtained by this step exhibited a nearly pure polypeptide by silver staining after SDS-PAGE. We calculated the recovery of the receptor protein to be about 28%, resulting in about a 1400-fold purification in this step. The series of purification procedures finally resulted in a *ca.* 13 400-fold purification of the receptor protein, with a recovery and specific activity of 9% and 5400 pmol/mg of protein, respectively (Table I).

DISCUSSION

The polypeptide of $M_r \approx 94\,000$, assessed by SDS-PAGE (10% acrylamide gel), was photolabelled with [^{125}I]azido-NAPS, with an appropriate pharmacological profile of D_2 dopamine receptor. The relative mobility of this photolabelled D_2

dopamine receptor on SDS-PAGE varied when the acrylamide concentration of the separation gel was changed from 6% to 12%. The apparent molecular weight of the polypeptide at each acrylamide concentration was calculated to be *ca.* 78 000 (6%), 88 000 (7.5%), 94 000 (10%) and 130 000 (12%). The D_2 dopamine receptor has an affinity to wheat germ lectin[15]. Sequential exoglycosidase (neuraminidase)–endoglycosidase (glycopeptidase-F) treatment altered the electrophoretic mobility of the 94-kDa labelled band to *ca.* 43 kDa (data not shown)[11,12,16]. Therefore, the D_2 dopamine receptor protein obviously contains an aspartate-linked polyglycoside chain. The decrease in mobility on SDS-PAGE seemed to be caused by a large polyglycoside chain of D_2 dopamine receptor. The exact reason for the variation of the mobility that was observed on changing the acrylamide concentration is still unclear, but it seems that changes in hydration and steric structure contribute to this phenomenon. Recently, it was reported that an opiate receptor, which also has an affinity to wheat germ lectin, exhibited a similar strange mobility on SDS-PAGE[17].

We utilized this unusual character for the purification of D_2 dopamine receptor using preparative SDS-PAGE. In order to solve the problem of the limited volume of loading in SDS-PAGE, we employed partial purification steps with gel permeation HPLC and hydroxyapatite HPLC prior to the preparative SDS-PAGE. Bovine synaptic membrane was solubilized by SDS, subjected to gel permeation HPLC and hydroxyapatite HPLC and separated by sequential SDS-PAGE (6% acrylamide gel and 12% acrylamide gel; 3-mm thick slab gel). This procedure resulted in about a 13 400-fold purification with about 9% recovery. Further, D_2 dopamine receptor protein was detected as an apparent single polypeptide, assessed by silver staining and autoradiography after SDS-PAGE. The degree of purification of the D_2 dopamine receptor by our procedure is similar to those reported recently by the other workers[6,7].

The direct detection of the binding activity of purified receptor protein is difficult because of the presence of SDS in all purification steps. However, we concluded that the purified polypeptide is the D_2 dopamine receptor, because we could observe the photolabelled band with [^{125}I]azido-NAPS, which was blocked by several drugs with an appropriate pharmacological profile, at the same position by autoradiography as that shown by silver staining. [^{125}I]Azido-NAPS is a specific antagonist to D_2 dopamine receptor.

We would emphasize the advantages of this method over previous purification procedures that it is simple and reproducible and we could obtain high recoveries of receptor protein. The general procedure for the purification of receptor protein is as follows: the receptor protein is solubilized with mild detergent while holding an intact ligand binding activity, and then applied to ligand-affinity chromatography and to fixed lectin-affinity chromatography. However, with ligand-affinity chromatography, which is a highly efficient procedure for receptor purification, it is difficult to obtain a reproducible result. We attempted the ligand-affinity chromatography [with a haloperidol-Sepharose column, carboxymethyleneoximinospiperone(CMOS)-Sepharose column, etc.] according to several previous reports[3,5,7], but could not obtain satisfactory results. This is probably due to the difficulty in finding the exact conditions necessary to exclude non-specific absorption on an affinity column. Moreover, even if the receptor protein was successfully purified to a single polypeptide according to these procedures, the recovery of receptor protein was usually below 1%. Therefore, it is difficult to prepare a sufficient amount of purified receptor protein for determination of the amino acid sequence by the procedures reported previously.

The purification procedure described here is simple and efficient in comparison with previous purification procedures. If necessary, we can use an additional step of reversed-phase HPLC with an acidic mobile phase, which seems to be effective for the separation of the membrane proteins. The purified protein obtained by this method did not have ligand-binding activity, but can be used for the determination of the amino acid sequence of this protein. When the partial amino acid sequence of the 94-kDa protein was determined, the cloning and expression of cDNA for this protein also seemed possible in the animal cell. The primary amino acid sequence and the characterization of the expressed protein in the animal cell will confirm that the purified 94-kDa protein is indeed the D_2 dopamine receptor. A similar purification procedure, using photolabelled receptor protein as a tracer, should be useful for the receptor proteins other than D_2 dopamine receptor.

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