Affinity Chromatography of the D_1 Dopamine Receptor from Rat Corpus Striatum†

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ABSTRACT: The D_1 dopamine receptor from rat corpus striatum has been purified 200–250-fold by using a newly developed biospecific affinity chromatography matrix based on a derivative of the D_1 selective antagonist SCH 23390. This compound, (*RS*)-5-(4-aminophenyl)-8-chloro-2,3,4,5-tetrahydro-3-methyl-1*H*-3-benzazepin-7-ol (SCH 39111), possesses high affinity for the D_1 receptor and, when immobilized on Sepharose 6B through an extended spacer arm, was able to adsorb digitonin-solubilized D_1 receptors. The interaction between the solubilized receptor and the affinity matrix was biospecific. Adsorption of receptor activity could be blocked in a stereoselective fashion [SCH 23390 > SCH 23388; (+)-butaclamol > (−)-butaclamol]. The elution of [^{3}H]SCH 23390 activity from the gel demonstrated similar stereoselectivity for antagonist ligands. Agonists eluted receptor activity with a rank order of potency consistent with that of a D_1 receptor [apomorphine > dopamine > (−)-epinephrine ≫ LY 171555 > serotonin]. SCH 39111–Sepharose absorbed 75–85% of the soluble receptor activity, and after the gel was washed extensively, 35–55% of the absorbed receptor activity could be eluted with 100 μM (+)-butaclamol with specific activities ranging from 250 to 450 pmol/mg of protein. The affinity-purified receptor retains the ligand binding characteristics of a D_1 dopamine receptor. This affinity chromatography procedure should prove valuable in the isolation and molecular characterization of the D_1 dopamine receptor.

Dopamine exerts physiologic effects in both the central nervous system and the periphery, and these effects are mediated through two receptor subtypes: D_1 and D_2 [for reviews, see Kebabian and Calne (1979) and Stoof and Kebabian (1984)]. Dopamine systems are implicated in several devastating disease processes including schizophrenia, Parkinson's disease, and possibly Huntington's chorea [for review, see Seeman (1987)]. In the periphery, dopamine acting through the D_1 receptor is known to stimulate the release of parathyroid hormone (Brown et al., 1977, 1980; Attie et al., 1980), promote renal vasodilatation [for review, see Kohli and Goldberg (1987)], and control gap junction permeability in electrically coupled cells of the retina (Neyton et al., 1985; Lasater & Dowling, 1985). In the central nervous system, a physiologic role for the D_1 receptor has been more difficult to define. Until recently, almost all of the effects of dopamine in the central nervous system were thought to be mediated through the D_2 receptor, including antipsychotic effects of neuroleptic medications, antistereotyptic effects, cataleptogenic effects, and avoidance inhibition [for reviews, see Seeman (1980) and Arnt (1987)]. However, studies that examined the behavioral effects of the first D_1 selective antagonist, SCH 23390, found this compound to elicit many of the same effects expected of a D_2 antagonist (Iorio et al., 1983; Christensen et al., 1984; Mailman et al., 1984; Waddington, 1986). These unexpected findings have rekindled interest in defining the functional role of the D_1 receptor in the central nervous system.

The D_1 receptor is thought to mediate its effects entirely through the stimulation of the adenylyl cyclase enzyme (Kebabian & Calne, 1979) and subsequent activation of the cAMP-dependent protein kinase (Hemmings et al., 1987). An understanding of the mechanisms by which the D_1 receptor mediates its effects in the central nervous system and the periphery will require the purification of the receptor and the study of its interactions with the other components involved in its signal transduction pathway.

Although the ligand binding subunit of the D_1 receptor has been identified by photoaffinity cross-linking (M_r 72 000; Amlaiky et al., 1987), only limited progress toward the solubilization and purification of this receptor has been previously reported. The D_1 receptor from rat striatum has been solubilized with the detergent cholate (Sidhu & Fishman, 1986). However, this procedure required the removal of detergent by SM2 Bio-Beads before ligand binding could be measured. The D_1 receptor has been solubilized with digitonin from canine and bovine brain; however, the yield and specific activity that results from these tissues were low, and early attempts to purify the D_1 receptor yielded only minimal improvements in specific activity (Niznik et al., 1986a,b; Dumbrille-Ross et al., 1985). Although a preliminary report of a potential affinity ligand for the D_1 receptor has appeared recently (Wray et al., 1987), its usefulness has not been documented. In this paper, we describe the synthesis of a new affinity ligand and its use in the development of a biospecific affinity chromatography procedure for the D_1 dopamine receptor. In a single step, this procedure yields a 200–250-fold purification of the receptor ligand binding subunit from rat corpus striatum to a specific activity of 250–450 pmol/mg of protein. This affinity chromatography matrix should prove valuable for both the complete purification of this receptor and its subsequent biochemical characterization.

Materials and Methods

Materials. The following materials were obtained from Sigma (St. Louis, MO): epoxy-activated Sepharose 6B, HEPES,[1] Tris base, pepstatin A, leupeptin, soybean trypsin

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inhibitor, aprotinin, α_1-antitrypsin, PMSF, EDTA, EGTA, (−)-epinephrine, serotonin, NaCl, and sucrose. 1,6-Diaminohexane and ethanolamine were from Aldrich (St. Louis, MO); EDAC was obtained from Bio-Rad (Richmond, CA); frozen rat brains were from Pel-Freeze Biologicals (Rogers, AR); cholic acid was from Kodak Biochemicals (Rochester, NY); Sephadex G-50 was from Pharmacia (Uppsala, Sweden); (+)-butaclamol, apomorphine, and dopamine were obtained from RBI (Natick, MA); the other drugs in this study were generous gifts from previously named sources (Caron et al., 1978); digitonin was from WAKO Chemicals (Dallas, TX); [^{3}H]SCH 23390 was from Amersham (Arlington Heights, IL).

Chemical Syntheses. (*A*) *SCH 39111–Succinic Anhydride Adduct.* A solution of succinic anhydride (0.35 g, 3.5 mmol) and SCH 39111 (1.0 g, 3.3 mmol) in 25 mL of dimethylformamide (DMF) was stirred at 40 °C for 2 h and then poured into 200 mL of ice water. The solid that gradually crystallized from the mixture was filtered, washed with water, and dried in vacuo to give 0.91 g of product: mp 259–260 °C dec; mass spectrum (FAB) $(M + H)^+$ 403.1434, calculated for $C_{21}H_{24}N_2O_4Cl$ 403.1425. Anal. Calcd for $C_{21}H_{23}N_2O_4Cl$: C, 62.61; H, 5.71; N, 6.96. Found: C, 61.08; H, 5.64; N, 6.81.

(*B*) (*RS*)-*5-(4-Aminophenyl)-8-chloro-3-methyl-2,3,4,5-tetrahydro-1H-3-benzazepin-7-ol* (*SCH 39111*). A solution of 1-[4-(acetylamino)phenyl]-7-chloro-8-methoxy-3-methyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (1.8 g) in 20 mL of 48% HBr was heated with stirring on an oil bath at 130–135 °C for 5 h. The mixture was concentrated under vacuum at 120 °C to ca. 5 mL, cooled, dissolved in 20 mL of water, and poured into a stirred mixture of 5 g of $NaHCO_3$ in 60 mL of water. Precipitated material was filtered and the aqueous filtrate concentrated to give additional solids. These were combined and recrystallized from 90% ethanol to give 1.1 g of product: mp 205–206 °C; ^{1}H NMR (Me_2SO-d_6) (79.5 MHz) δ 2.26 (3 H, s, NCH_3), 4.02 (1 H, d, J = 7 Hz, 5-H), 4.90 (2 H, br s, 4′-NH_2), 6.31 (1 H, s, 9-H), 6.66 (4 H, AB q, 2′-H, 3′-H, 5′-H, 6′-H), 7.06 (1 H, s, 6-H), 9.55 (1 H, s, 7-OH); mass spectrum (EI) M^+ 302, 304. Anal. Calcd for $C_{17}H_{19}N_2ClO$: C, 67.43; H, 6.34; N, 9.26. Found: C, 67.27; H, 6.22; N, 9.01.

(*C*) *1-[4-(Acetylamino)phenyl]-7-chloro-8-methoxy-3-methyl-2,3,4,5-tetrahydro-1H-3-benzazepine.* *N*-[2-Hydroxy-2-[4-(acetylamino)phenyl]ethyl]-*N*-methyl-3-chloro-4-methoxyphenethylamine (7.95 g) was treated with 80 mL of anhyrous methanesulfonic acid at 0 °C. The resulting mixture was then allowed to warm to room temperature and stirred overnight. It was then diluted with 600 mL of water and basified with Na_2CO_3. The resulting mixture was extracted twice with 200-mL portions of CH_2Cl_2, the extracts were combined and evaporated, and solid residue was recrystallized twice from ethyl acetate to give 1.85 g of product: mp 197–199 °C; mass spectrum (CI) $(M + H)^+$ 359, 361; ^{1}H NMR ($CDCl_3$) (79.5 MHz) δ 2.21 (3 H, s, NCH_3), 2.41 (3 H, s, OCH_3), 3.67 (3 H, s, $COCH_3$), 4.27 (1 H, dd, J = 3, 7 Hz, 1-H), 6.28 (1 H, s, 9-H).

(*D*) *N-[2-Hydroxy-2-[4-(acetylamino)phenyl]ethyl]-N-methyl-3-chloro-4-methoxyphenethylamine.* A mixture of *N*-[2-acetoxy-2-[4-(acetylamino)phenyl]ethyl-*N*-methyl-3-chloro-4-methoxyphenethylamine (10.5 g) in 150 mL of methanol and 75 mL of 1 N K_2CO_3 was heated on a steam bath with stirring for 1 h. The mixture was then concentrated to ca. 120 mL, diluted with 300 mL of water, and extracted with two 120-mL portions of ether. The combined extracts were dried over anhydrous K_2CO_3, filtered, and evaporated to dryness in vacuo to give 9.0 g of oily residue. A sample was crystallized from ethyl acetate, mp 107–108 °C. Anal. Calcd for $C_{20}H_{25}N_2O_5Cl$: C, 63.73; H, 6.69; N, 7.43. Found: C, 63.83; H, 6.69; N, 7.31.

(*E*) *N-[2-Acetoxy-2-[4-(acetylamino)phenyl]ethyl]-N-methyl-3-chloro-4-methoxyphenethylamine.* A mixture of *p*-nitrostyrene oxide (6.0 g) and *N*-methyl-3-chloro-4-methoxyphenethylamine (7.2 g) was heated in an oil bath at 135–140 °C with stirring for $3^1/_2$ h. The viscous reaction mixture was dissolved in a mixture of 300 mL of ethanol containing 13 mL of 37% HCl and hydrogenated over 1.5 g of 5% Pd/C. Uptake of hydrogen was very rapid and was stopped after absorption of 105% of theory. Catalyst was filtered, the filtrate was evaporated to dryness, and the residue was partitioned between 200 mL each of water and CH_2Cl_2. Na_2CO_3 solution was added until the aqueous layer was basic, and the organic layer was separated, dried over anhydrous K_2CO_3, and filtered. The filtrate was treated with 13 mL of acetic anhydride and 50 mg of 4-(*N*,*N*-dimethylamino)pyridine and stirred overnight. The reaction mixture was then treated with 20 mL of methanol, an additional 50 mg of 4-(*N*,*N*-dimethylamino)pyridine was added, and the resultant mixture was heated at reflux for 20 min. The mixture was chilled in ice and diluted with 200 mL of water, and solid Na_2CO_3 was added until basic. The organic layer was separated, dried over anhydrous K_2CO_3, filtered, and evaporated to dryness to give 14.5 g of oily product.

Solubilization of the D_1 Dopamine Receptor. Rat brains were thawed in a solution containing 50 mM HEPES (pH 7.2), 100 mM NaCl, 10 mM EDTA, 10 mM EGTA, and 250 mM sucrose (solution A). Striata were dissected and homogenized in 80 volumes of ice-cold solution A (containing the additional protease inhibitors 50 μM PMSF, 5 μg/mL each of pepstatin A, leupeptin, soybean trypsin inhibitor, and aprotinin, and 1 μg/mL α_1-antitrypsin), with 10 strokes in a Potter–Elvehjem homogenizer. The crude homogenate was pelleted by centrifugation for 30 min at 45000*g*. The pellets were resuspended in 80 volumes of solution A without sucrose but with protease inhibitor cocktail (solution B) and centrifuged for 20 min at 45000*g*. This step was repeated once more, and the resulting pellet was resuspended in 25 mL/g wet weight striatal tissue of solution B containing 1% (w/v) digitonin and allowed to stir slowly on ice for 60 min. The solubilized preparation was obtained by centrifugation for 90 min at 45000*g*. Longer centrifugation times or ultracentrifugation at 100000*g* did not pellet additional receptor activity.

Binding Assay for Solubilized Receptor. Solubilized receptor was assayed by using [^{3}H]SCH 23390 (78 Ci/mmol) in a volume of 0.25 mL containing 0.10 mL of soluble preparation and 0.15 mL of 0.1% digitonin, 50 mM HEPES (pH 7.2), 100 mM NaCl, 5 mM EDTA (solution C), and 10 nM [^{3}H]SCH 23390. Nonspecific binding was defined by the inclusion of 4 μM SCH 23390. Samples were incubated for either 2 h at 22 °C or 16 h at 4 °C. Both conditions yielded equivalent results. Bound and free radioligands were separated by gel filtration (Sephadex G-50, fine) on 0.6 × 13.5 cm columns equilibrated with 0.1% cholate, 50 mM Tris (pH 7.0, 22 °C), 120 mM NaCl, and 2.5 mM EDTA as described by

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; FAB, fast atom bombardment; EI, electron impact; CI, chemical ionization; GppNHp, 5′-guanylyl imidodiphosphate; LY 171555, quinpirole; Tris, tris(hydroxymethyl)aminomethane.

Caron et al. (1979). Nonspecific binding was typically less than 0.25% of the total added radioactivity and less than 5% of bound.

Synthesis of SCH 39111–Sepharose. Epoxy-Sepharose 6B containing 15–20 μmol/mL derivatized 1,4-butanediol diglycidyl ether was rehydrated, washed extensively with water, and equilibrated with 0.1 M $NaHCO_3$. The moist gel cake was resuspended (2 mL/mL of gel) in a solution containing 0.1 M $NaHCO_3$, 0.05 M 1,6-diaminohexane, and 0.95 M ethanolamine, stirred slowly at room temperature for 4 h, and finally washed extensively with water. The amino-activated epoxy-Sepharose 6B was gradually equilibrated with 100% dimethyl sulfoxide (DMSO). SCH 39111–succinate (0.25 mg/mL of gel) was dissolved in DMSO (1 mg/mL) with gentle heating to 55 °C and added to the amino-activated epoxy-Sepharose 6B, and the final volume was brought to 2 mL of DMSO/mL of gel. EDAC was added to this slurry (50 mg/mL of gel), and the slurry was slowly stirred at 22 °C for 24 h. The ligand-coupled resin was washed with DMSO over a period of 12 h, at which time the wash solution was gradually brought to 100% water by stepwise increments over a 6-h period. The resulting gel was finally washed with water and stored at 4 °C.

Affinity Chromatography of the Solubilized D_1 Dopamine Receptor. Typically, 50 mL of solubilized preparation was adsorbed batchwise (ca. 75 pmol of [^{3}H]SCH 23390 binding sites) by incubation with 5 mL of SCH 39111–Sepharose with slow rotation for 20 h at 4 °C. The resin was collected in a 1.5 cm diameter column and washed with the following series of solutions at 4 °C: 5 bed volumes of solution C; 5 bed volumes of solution C containing 1% digitonin and 250 mM NaCl; 5 bed volumes of solution C containing 500 mM NaCl; 5 bed volumes of solution C; and finally 5 bed volumes of solution C (pH 6.0) at 22 °C.

The resin was then eluted with solution C (pH 6.0) containing 100 μM (+)-butaclamol dissolved in methanol (final methanol concentration = 0.01%) at 22 °C with a flow rate of 5 mL/h over a period of 6 h, collecting fractions at 30-min intervals. The eluates were collected on ice in tubes containing an equal volume of solution C (pH 7.2) to readjust the pH of the eluate to 6.8. The final two eluates were collected at the same flow rate but with 3 h per fraction. In order to assay eluted activity, the eluate was desalted on a Sephadex G-50 (fine) column (0.6 × 13.5 cm) to separate unbound (+)-butaclamol from the receptor. The desalted eluate was then incubated with 10 nM [^{3}H]SCH 23390 and assayed for soluble binding.

Protein Determinations. Protein concentrations in solutions containing more than 1 μg of protein/mL were determined by the method of Bradford (1976) using bovine serum albumin as a standard. Solutions containing less than 1 μg of protein/mL were assayed by using the Amido-Schwarz method of Schaffner and Weissman (1973).

Results and Discussion

Solubilization of D_1 Dopamine Receptor from Rat Striatum. Membrane homogenates made from rat striatal tissue as described under Materials and Methods contained 120 pmol of [^{3}H]SCH 23390 binding sites/g of tissue (1.8 pmol/mg of protein). Treatment of striatal membranes with 1% digitonin under the conditions specified under Materials and Methods solubilized 35–40% of these binding sites, yielding preparations containing ca. 1.7–1.9 pmol/mL (specific activity ca. 1.5–1.7 pmol/mg of protein). Occupying the receptor with an agonist such as SKF 38393 prior to solubilization has been reported to improve receptor recovery during solubilization (Sidhu & Fishman, 1986), but in this case, agonist pretreatment afforded no improvement in recovery from membranes (data not shown). Incubation with 10 nM [^{3}H]SCH 23390 prior to solubilization improved the recovery marginally (data not shown); however, due to the difficulty of removing such a high-affinity ligand in order to allow subsequent affinity chromatography, such a step was not routinely employed.

Table I: Ligand Binding Properties of Soluble and Affinity-Purified D_1 Receptor Preparations[a]

	K_D (nM)	
	soluble	affinity purified
antagonists		
SCH 23390	1	5
SCH 23388	94	1220
(+)-butaclamol	154	940
(−)-butaclamol	>42000	16890
cis-flupenthixol	65	40
trans-flupenthixol	11300	4120
piflutixol	5	11
fluphenazine	250	630
promethazine	12500	44000
sulpiride	>46000	313000
ketanserin	3468	8800
yohimbine	>100000	166700
prazosin	>100000	>100000
propranolol	194000	142000
agonists		
SKF 38393	7	22
apomorphine	56	325
dopamine	140	470
(−)-epinephrine	1366	10420
LY 171555	384000	120000
serotonin	114000	445000

[a] Competition experiments were performed as described under Materials and Methods, using [^{3}H]SCH 23390 (4–5 nM) for solubilized and [^{125}I]SCH 23982 (1–3 nM) for affinity-purified preparations. K_D values for the radioligands were determined by direct saturation experiments and isotope dilution experiments with nonradiolabeled SCH 23390 and SCH 23982 (data not shown). Each method yielded equivalent results (K_D for [^{3}H]SCH 23390 = 1.0 nM for the solubilized preparation and K_D for [^{125}I]SCH 23982 = 12 nM for affinity-purified preparations). The data were analyzed by using computer modeling methods previously described (De Lean et al., 1982) and are the average of two independent determinations whose pK_D values were within 10%. K_D values that show a greater than sign (>) are curves that could not be modeled because of their low affinity, and the value shown represents the lowest K_D estimate for that experiment.

The solubilized binding sites retained the pharmacologic properties of the D_1 receptor ligand binding subunit (see Table I). Soluble receptor preparations exhibited high affinity for SCH 23390, piflutixol, and *cis*-flupenthixol and much lower affinity for less active compounds such as fluphenazine and promethazine. The stereoselective properties of the receptor were also preserved upon solubilization [SCH 23390 (*R*) > SCH 23388 (*S*); *cis*-flupenthixol ≫ *trans*-flupenthixol; (+)-butaclamol ≫ (−)-butaclamol]. The specificity of the soluble receptor was demonstrated by the very low affinity found for ligands selective for other receptor types (e.g., D_2 dopaminergic, sulpiride; S_2 serotonergic, ketanserin; α_2 adrenergic, idazoxan; α_1 adrenergic, prazosin; β adrenergic, propranolol). Agonist binding was also consistent with that expected for the D_1 receptor [SKF 38393 > apomorphine > dopamine ≫ (−)-epinephrine ≫ LY 171555 > serotonin].

Synthesis of SCH 39111–Sepharose. The synthesis of SCH 39111 is described under Materials and Methods. SCH 39111 is a closely related structural analogue of the highly selective and potent D_1 dopamine antagonist SCH 23390; however, SCH 39111 possesses an amine moiety on the para position of the 1-phenyl ring (see Figure 1A) and retains very high affinity for the D_1 dopamine receptor (2.5 nM in membranes,

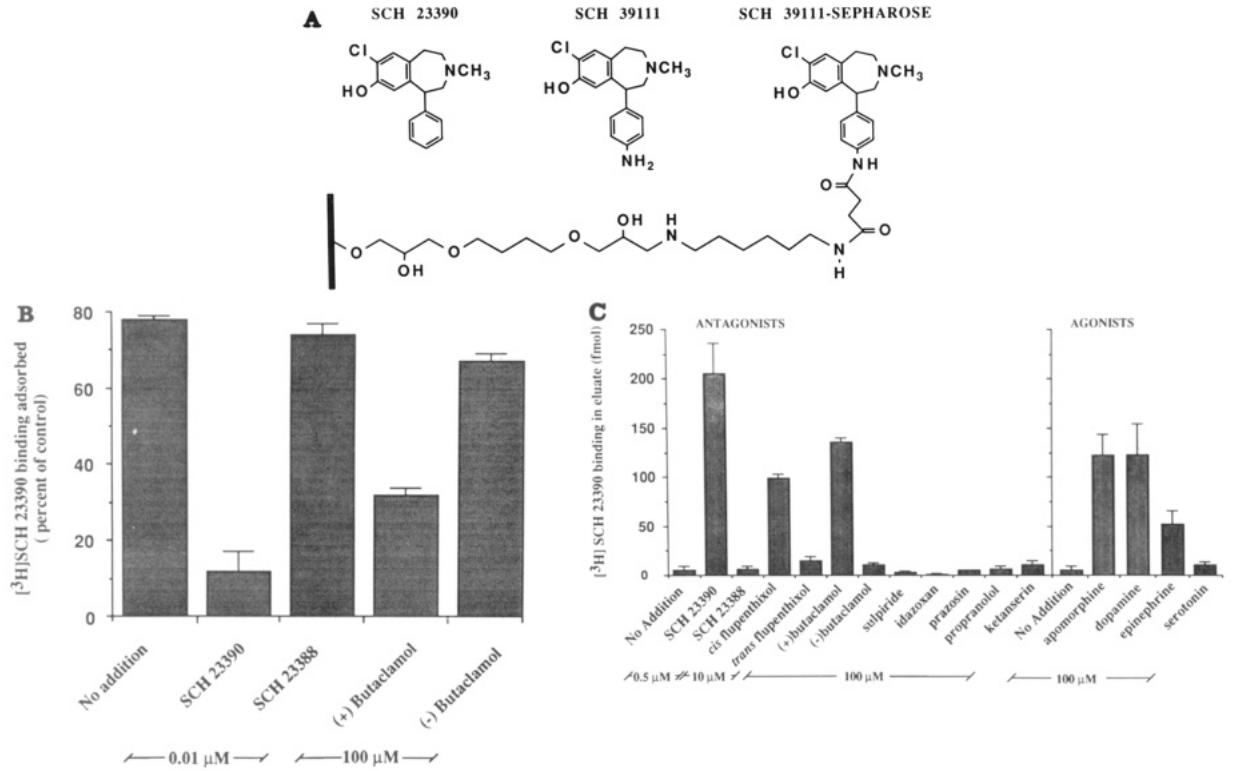

FIGURE 1: Structure and biospecific characteristics of SCH 39111–Sepharose. (A) Structures of SCH 23390, SCH 39111, and the presumed structure of SCH 39111–Sepharose: SCH 23390 has been demonstrated to be a high-affinity and selective ligand for the D_1 dopamine receptor. SCH 39111 was synthesized in order to allow immobilization to a solid support matrix with preservation of its affinity and D_1 selective properties. SCH 39111 was immobilized to Sepharose 6B through the extended spacer arm shown. (B) Biospecificity of adsorption: 1-mL aliquots of the digitonin-solubilized receptor preparation were incubated with the indicated concentrations of pharmacologic agents for 1 h at 22 °C, at which time 0.25 g of a moist gel cake of SCH 39111–Sepharose was added to the sample. The resulting slurry was mixed by slow rotation for 20 h at 4 °C. The affinity gel was allowed to settle and 0.5 mL of supernatant was desalted by Sephadex G-50 gel filtration and assayed for [^{3}H]SCH 23390 binding activity as described under Materials and Methods. The histogram bars indicate the percent of binding activity adsorbed by SCH 39111–Sepharose in the presence of the indicated agents expressed as a percentage of original soluble binding activity. The results shown are the average of two experiments. The error bars reflect the range of the two values determined in duplicate. (C) Biospecificity of elution: Digitonin-solubilized receptor was incubated with SCH 39111–Sepharose (10 mL of soluble/mL of resin) for 20 h at 4 °C. The resin was then washed with 10 bed volumes of solution C, 10 bed volumes of this same solution containing 500 mM NaCl, 10 bed volumes of solution C, and 10 bed volumes of solution C but with pH 6.0 at 22 °C and then resuspended in solution C, pH 6.0 (1 mL/original mL of soluble preparation), containing the indicated concentration of the pharmacologic agent, and the slurry was rotated slowly at 22 °C for 5 h. The resin was allowed to settle and 0.5 mL of the supernatant was desalted by Sephadex G-50 gel filtration and assayed for [^{3}H]SCH 23390 binding as described under Materials and Methods. The results shown are the averages of two to three experiments. The error bars reflect the range of values obtained for all determinations. In the case of SCH 23390 elution, the receptor was eluted with the tritiated ligand whose specific activity was diluted with unlabeled ligand. This was necessary due to the difficulty of removing SCH 23390 from the receptor by gel filtration.

8.3 nM in solubilized preparations; data not shown). In order to immobilize SCH 39111 to a suitable matrix, the ligand was first treated with succinic anhydride, creating a short four-carbon chain terminating in a carboxylic acid group. Sepharose reacted with 1,4-butanediol diglycidyl ether to create an epoxy-activated support was further treated with 1,6-diaminohexane to create an extended spacer arm terminating in an amino group. The succinylated derivative of SCH 39111 was then coupled to this amino-activated support by using the water-soluble carbodiimide, EDAC. Ethanolamine was included to deactivate the excess epoxy groups. The presumed structure of this matrix is shown in Figure 1A.

Biospecific Characteristics of SCH 39111–Sepharose. SCH 39111–Sepharose adsorbed and allowed elution of [^{3}H]SCH 23390 binding activity in a biospecific fashion, demonstrating that the immobilized derivative of SCH 39111 maintained the characteristics of a D_1 selective ligand. As demonstrated in Figure 1B, incubation of solubilized preparations with D_1 dopamine antagonists such as SCH 23390 or (+)-butaclamol prior to exposure to the affinity resin reduced adsorption of [^{3}H]SCH 23390 binding activity by 90 and 70%, respectively. The less active stereoisomers, SCH 23388 and (−)-butaclamol, resulted in only a modest reduction of the adsorption observed with no ligand present.

The biospecificity of SCH 39111–Sepharose was further demonstrated during the elution of adsorbed receptor activity. As seen in Figure 1C, the D_1 dopamine receptor antagonists, SCH 23390, *cis*-flupenthixol, and (+)-butaclamol, eluted receptor activity from the affinity matrix, while the less potent stereoisomers, SCH 23388, *trans*-flupenthixol, and (−)-butaclamol, failed to elute significantly more receptor activity than with no added ligand. Dopaminergic agonists eluted receptor with a rank order of potency expected for a D_1 dopamine receptor [apomorphine ≥ dopamine > (−)-epinephrine ≫ serotonin]. Furthermore, several selective and potent antagonists at other catecholamine and serotonergic receptors also failed to elute significant amounts of [^{3}H]SCH 23390 binding activity (Figure 1C).

Chromatography of Digitonin-Solubilized Striatal Membranes on SCH 39111–Sepharose. Batchwise incubation of digitonin-solubilized membranes with SCH 39111–Sepharose (typically 10 mL of soluble preparation/mL of resin) resulted in the adsorption of 75–85% of the [^{3}H]SCH 23390 binding activity, while the bulk of the protein (ca. 90%) was not retained on the matrix. As shown in Figure 2, washing the gel with solutions containing various concentrations of salt and

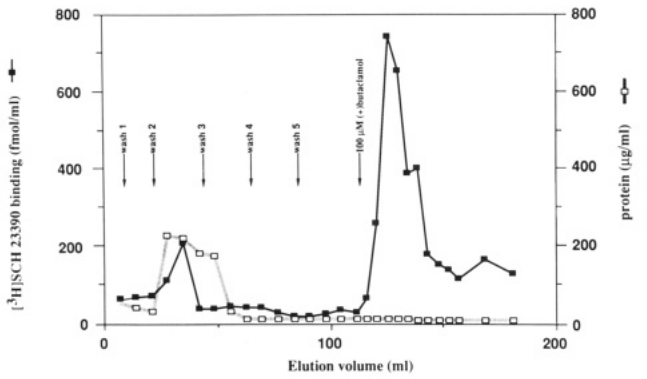

FIGURE 2: Elution profile of SCH 39111–Sepharose. Digitonin-solubilized membranes (45 mL) were batch adsorbed to 4.5 mL of SCH 39111–Sepharose by slowly mixing at 4 °C for 20 h, and then the gel slurry was washed (1 mL/min) on a column (1.5 cm) as described under Materials and Methods. The arrows indicate the beginning of each washing step: wash 1, solution B, pH 7.2, 4 °C; wash 2, solution B containing 1% digitonin and 250 mM NaCl, pH 7.2, 4 °C; wash 3, solution B containing 500 mM NaCl, pH 7.2, 4 °C; wash 4, solution B, pH 7.2, 4 °C; wash 5, solution B, pH 6.0, 22 °C. The last arrow marks the beginning of the elution at which point the flow rate was decreased to 5 mL/h. The column was eluted with solution B (pH 6.0, 22 °C) containing 100 μM (+)-butaclamol in methanol (final MeOH concentration = 0.01%). Eluates were then collected over the next 12 h, desalted to remove unbound (+)-butaclamol, and assayed for [^{3}H]SCH 23390 binding activity. Protein in the wash fractions was assayed by using the method of Bradford; protein in the eluates was measured by the Amido-Schwarz method. The stippled line (open symbols) represents the elution of protein from the affinity matrix in μg/mL. The solid line (filled symbols) indicates [^{3}H]SCH 23390 binding activity in each fraction expressed as fmol/mL. The horizontal axis represents the total volume of the elution profile. This profile is a representative example of eight separate experiments.

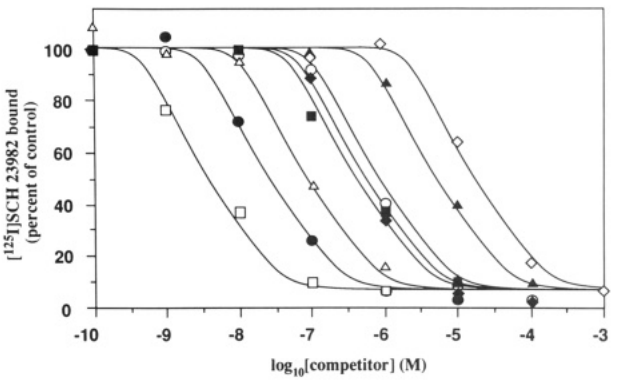

FIGURE 3: Pharmacological properties of ligand binding to affinity-purified D_1 dopamine receptors. The eluted receptor was pooled and concentrated by using an Amicon concentrator with a YM-30 membrane. The concentrated receptor was desalted on Sephadex G-50 gel filtration columns equilibrated with 0.1% digitonin solution to remove unbound (+)-butaclamol remaining after elution. Aliquots of this material (0.075 mL) were incubated with 1–3 nM [^{125}I]SCH 23982 (K_D = 12 nM, by direct saturation experiments and isotope dilution experiments with nonradiolabeled SCH 23982; data not shown) and the indicated concentration of the competing ligand in a total volume of 0.10 mL for 2 h at 22 °C. Bound and free ligands were separated on Sephadex G-50 gel filtration columns as described under Materials and Methods. Incubations containing agonist ligands also contained 100 μM sodium metabisulfite as an antioxidant. Symbols: (□) SCH 23390, (■) SCH 23388, (△) *cis*-flupenthixol, (▲) *trans*-flupenthixol, (●) SKF 38393, (◆) apomorphine, (○) dopamine, and (◇) (−)-epinephrine.

detergent (arrows) removed most of the protein while eluting little receptor activity (typically less than 15%). Biospecific elution of the receptor activity was achieved by addition of 100 μM (+)-butaclamol to the eluting solution. (+)-Butaclamol was used as the eluting ligand because of its ease of removal from the receptor by Sephadex G-50 gel filtration. The best recovery of receptor activity was obtained when the elution procedure was performed at room temperature and the pH of the eluting solution was lowered to 6.0. These conditions were found to maximize receptor dissociation from the affinity matrix and produced the best recovery of receptor activity. With this procedure, 35–55% of the adsorbed receptor activity could be recovered in the eluate with a resulting 200–250-fold purification (300–375 pmol/mg of protein) compared to the starting digitonin-solubilized preparation (1.5 pmol/mg of protein in this experiment). The resulting specific activity indicates that the affinity-purified receptor is only about 40-fold short of theoretical specific activity (13 889 pmol/mg of protein based on a M_r of 72 000; Amlaiky et al., 1987) or about 2–3% pure. It should be noted that the measurement of receptor activity is based upon assays of affinity-purified material at 10 nM [^{3}H]SCH 23390, a concentration of radioligand that would not be saturating for the affinity-purified material (see Table I); thus, the recovery and specific activity reported here may be slightly underestimated.

Characterization of the Ligand Binding Properties of the Affinity-Purified D_1 Receptor. As discussed above, both the solubilized receptor and the affinity matrix displayed the expected pharmacologic properties of a D_1 dopamine receptor and a D_1 ligand, respectively. To further document the identity of the partially purified [^{3}H]SCH 23390 binding activity isolated in this procedure, the ligand binding pharmacology of the affinity-purified preparation was characterized. For this purpose, a radioiodinated analogue of SCH 23390 was used. This compound, [^{125}I]SCH 23982, has been shown to possess high affinity and pharmacologic characteristics of a selective D_1 dopamine receptor ligand (Sidhu & Kebabian, 1985). The results of competition binding experiments with affinity-purified receptor using this ligand are shown in Figure 3 and summarized in Table I. Comparison of the dissociation constants obtained in affinity-purified preparations with those found for solubilized preparations demonstrates that the ligand binding properties of the receptor remain intact during the purification procedure, including stereoselectivity, receptor specificity, and agonist rank order of potency. The absolute affinities for almost all antagonists are decreased by 5–10-fold during solubilization, while the affinities measured for agonists in solubilized and affinity-purified preparations were higher than those reported in membrane-bound preparations (Billard et al., 1984; Andersen et al., 1985). However, these changes in agonist and antagonist affinities upon solubilization are not unprecedented as they are also observed in other soluble receptor preparations (Kilpatrick & Caron, 1983; Benovic et al., 1984; Lomasney et al., 1986).

In this procedure, we have relied exclusively on the ligand binding properties of the purified preparation to identify it as the D_1 receptor, and on this basis it is clear that a protein has been partially purified which has the expected properties of the ligand binding subunit of the D_1 dopamine receptor. Recent reports in the literature, however, have suggested that SCH 23390 and other antagonists might be recognizing a distinct form of the D_1 receptor that is not coupled to adenylyl cyclase stimulation (Anderson & Braestrup, 1986). Availability of pure receptor preparations will allow reconstitution experiments to determine whether the D_1 receptor ligand binding subunit isolated by this procedure can confer dopaminergic sensitivity to adenylyl cyclase.

Conclusions

In this paper, we describe an affinity chromatography procedure for the purification of the ligand binding subunit

of the D_1 dopamine receptor from rat corpus striatum. Several lines of evidence establish the D_1 receptor selectivity of SCH 39111–Sepharose. First, SCH 39111 has high affinity for D_1 receptors. SCH 39111, as a free ligand, potently displaces [^{3}H]SCH 23390 in both membranes and soluble preparations (data not shown). Second, the affinity matrix preferentially adsorbs the D_1 receptor relative to other proteins. Specifically, SCH 39111–Sepharose retains the bulk of SCH 23390 binding sites, while the vast majority of the protein in the preparation is unretarded. Third, the adsorption and elution of [^{3}H]SCH 23390 binding activity demonstrate the pharmacologic specificity expected for an interaction between a D_1 selective ligand and its receptor.

This affinity chromatography procedure substantially enriches D_1 dopamine receptor binding activity in a single chromatographic step, yielding preparations that are within 30–50-fold of theoretical specific activity. The affinity-purified receptor maintains the ligand binding characteristics of the membrane-bound receptor. The procedure described herein should allow the eventual complete purification of the D_1 dopamine receptor protein in sufficient quantities to permit the properties of the D_1 receptor and its biochemical coupling to be studied at the molecular level.

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