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Preparation of an affinity chromatography matrix for the selective purification of the dopamine D2 receptor from bovine striatal membranes

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A ligand affinity matrix has been developed and utilized to purify the dopamine D2 receptor approx. 2100 fold from bovine striatal membranes. 3-[2-Aminoethyl]-8-[3-(4-fluorobenzoyl)propyl]-4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (AES) was synthesized and used to prepare the affinity matrix by coupling to epoxy-activated Sepharose 6B (AES-Sepharose). AES ($K_i \approx 1.7$ nM) is similar in potency to the parent compound, spiperone ($K_i \approx 0.8$ nM), in competing for [^{3}H]spiperone-binding activity. AES has no significant potency in competing for the dopamine D1 receptor as assessed by competition for [^{3}H]SCH23390 binding ($K_i > 1$ μM). Covalent photoaffinity labeling of the dopamine D2 receptor in bovine striatal membranes with *N*-(*p*-azido-*m*-[^{125}I]iodophenethyl)spiperone ([^{125}I]N_3-NAPS) was prevented by AES at nanomolar concentrations. The dopamine D2 receptor was solubilized from bovine striatal membranes using 0.25% cholate in the presence of high ionic strength, followed by precipitation and subsequent treatment with 0.5% digitonin. Nearly 100% of the [^{3}H]spiperone-binding activity in the cholate-digitonin solubilized preparation was adsorbed at a receptor-to-resin ratio of 2:1 (v/v). Dopamine D2 receptor was eluted from the affinity resin using a competing dopaminergic antagonist molecule, haloperidol. Recovery of dopamine D2 receptor activity from the affinity matrix was approx. 9% of the activity adsorbed to the resin. The [^{3}H]spiperone-binding activity in AES-Sepharose affinity purified preparations is saturable and of high affinity (0.2 nM). Affinity-purified preparations maintain the ligand-binding characteristics of a dopamine D2 receptor as assessed by agonist and antagonist competition for [^{3}H]spiperone binding.

Introduction

Receptors for dopamine have been categorized as D1 or D2 receptors based on their biochemical function, pharmacological profile and tissue location [1–9]. Photoaffinity-labeling studies have revealed that the ligand-binding portions of D1 [10,11] and D2 [12–16] receptors reside on peptides of distinct molecular weights. Molecular cloning and expression of the rat-brain dopamine D2 receptor cDNA has been reported [17]. The cDNA codes for a 415 amino-acid peptide with a calculated relative molecular mass (M_r) of 47064. The receptor peptide is heavily glycosylated to form the mature protein with average $M_r \approx 92000$ [18–20]. The dopamine D2 receptor has been solubilized from both striatal and pituitary membranes of a variety of species, and the subject has recently been reviewed [3]. A number of different ligand affinity resins have been described for the purification of dopamine D2 receptors [21–27]. This report describes the development of a novel ligand affinity matrix based on the coupling of the butyrophenone antagonist, AES, to epoxy-activated Sepharose 6B. The affinity matrix, AES-Sepharose, has been utilized to affinity purify the dopamine D2 receptor from a preparation of solubilized bovine striatal membranes.

Abbreviations: AES, 3-[2-aminoethyl]-8-[3-(4-fluorobenzoyl)propyl]-4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one; [^{125}I]N_3-NAPS, *N*-(*p*-azido-*m*-[^{125}I]iodophenethyl)spiperone; DMF, dimethylformamide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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Materials and Methods

Materials

[^{3}H]Spiperone (21 Ci/mmol) was obtained from New England Nuclear (Boston, MA). *N*-(*p*-Azido-*m*-[^{125}I]iodophenethyl)spiperone ([^{125}I]N_3-NAPS) (2200

Ci/mmol) was generously provided by New England Nuclear. Epoxy-activated Sepharose 6B was purchased from Pharmacia (Piscataway, NJ). Digitonin was obtained from Gallard-Schlesinger (Carle Place, NY). Cholic acid and haloperidol were purchased from Sigma (St. Louis, MO). Whatman GF/B filters were obtained from Brandel (Gaithersburg, MD). All other chemicals were of reagent grade.

Synthesis of AES

2-(3-Chloropropyl)-2-(4-fluorophenyl)dioxolane (Fig. 1, product 1 [28]. A solution of 51 ml (0.31 mol) of 4-chloro-4′-fluorobutyrophenone, 19 ml (0.34 mol) of ethylene glycol, and 0.01 g of *p*-toluenesulfonic acid in 150 ml of benzene was heated to relux with azeotropic removal of water for 18 h. After cooling, the reaction was quenched by the addition of 50 ml of saturated aqueous sodium bicarbonate. The organic layer was separated and washed with 25 ml of saturated aqueous sodium chloride and dried with magnesium sulfate. Filtration and concentration under reduced pressure gave 70.4 g (92.9% of theoretical yield) of 2-(3-chloropropyl)-2-(4-fluorophenyl)dioxolane as an oil. NMR ($CDCl_3$) 1.8–1.9 (2H, m), 1.95–2.05 (2H, m), 3.5–3.6 (2H, t), 3.75–3.8 (2H, m), 4.0–4.05 (2H, m), 7.0–7.1 (2H, m), 7.4–7.5 (2H, m).

8-[3-(2-4′-Fluorophenyl-2-dioxolanyl)propyl]1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (Fig. 1, product 2). A mixture of 26.5 g (0.108 mol) of 2-(3-chloropropyl)-2-(4-fluorophenyl)dioxolane, 25.0 g (0.108 mol) of 1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one, 45.4 g (0.328 mol) of potassium carbonate, and 380 mg of potassium iodide in 200 ml of 4-methyl-2-pentanone was heated to reflux for 18 h. After cooling, the solution was filtered. The filtrate was concentrated under reduced pressure to give an oily residue that was redissolved in 200 ml of ethyl acetate. The organic solution was washed twice with 50 ml of water, once with 50 ml of saturated aqueous sodium chloride, and dried over magnesium sulfate. After filtration and concentration in vacuo, the residue was purified by flash chromatography on silica gel using ethyl acetate as the eluent. Collection of the major product gave 36.0 g (76% of theoretical yield) of 8-[3-(2-4′-fluorophenyl-2-dioxolanyl)propyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one as a solid; m.p. 149–150 °C. NMR ($CDCl_3$) 1.5–1.6 (2H, m), 1.65–1.75 (2H, br d), 1.85–1.95 (2H, m), 2.3–2.4 (2H, br t), 2.55–2.8 (6H, m), 3.7–3.8 (2H, m), 4.0–4.05 (2H, m), 4.73 (2H, s), 6.8–6.95 (3H, m), 6.95–7.05 (2H, m), 7.2 (1H, s), 7.25–7.3 (2H, m), 7.4–7.5 (2H, m).

N-t-Butyloxycarbonylethanolamine (Fig. 1, product 3). A solution of 21.8 g (0.10 mol) of di-*t*-butyldicarbonate in 100 ml of methylene chloride was added dropwise over 1 h to a cold (0 °C) solution of 6.0 ml (0.10 mol) of ethanolamine in 100 ml of methylene chloride. After the addition was complete, the reaction was allowed to warm to room temperature and then quenched by the addition of 50 ml of water. The organic phase was separated and washed successively with 50 ml each of saturated aqueous sodium bicarbonate, 1 M phosphoric acid, water and saturated aqueous sodium chloride. After drying over sodium sulfate, the solution was filtered and concentrated in vacuo to give 10.0 g (62.1% of theoretical yield) of the desired *N-t*-butyloxycarbonylethanolamine as an oil. NMR ($CDCl_3$) 1.45 (9H, s), 2.35 (1H, br s), 3.25–3.35 (2H, m), 3.71 (2H, t), 5.0 (1H, br s).

N-t-Butyloxycarbonylethanolamine-O-methanesulfonate (Fig. 1, product 4). A solution of 2.48 g (15.4 mmol) of *N-t*-butyloxycarbonylethanolamine and 3.22 ml (23.1 mmol) of triethylamine in 77 ml of methylene chloride was cooled to 0 °C and 1.37 ml (17.7 mmol) of methanesulfonyl chloride was added dropwise over 5 min. The reaction was stirred at 0 °C for 15 min and quenched by the addition of 25 ml of water. The organic phase was separated and washed successively with 25 ml each of saturated aqueous sodium bicarbonate, 1 M phosphoric acid, water and saturated aqueous sodium chloride. After drying over sodium sulfate, filtering, and concentrating under reduced pressure, 3.54 g (96.1% of theoretical yield) of *N-t*-butyloxycarbonylethanolamine-*O*-methanesulfonate was obtained as an oil. NMR ($CDCl_3$) 1.45 (9H, s), 2.7 (3H, s), 3.4 (2H, t), 4.0 (2H, t), 5.1 (1H, br s).

3-(2-N-t-Butyloxycarbonylethyl)-8-[3-(2-4′-fluorophenyl-2-dioxolanyl)propyl]-1-phenyl-1,3,8-triazaspiro-[4.5]decan-4-one (Fig. 1, product 5). 8-[3-(2-4′Fluorophenyl-2-dioxolanyl)propyl]-1-phenyl-1,3,8-triazaspiro-[4.5]decan-4-one (5.78 g, 13.2 mmol) was added as a solid to a mixture of 0.40 g (16.5 mmol) of sodium hydride in 10 ml of dimethylformamide which had been previously cooled to 0 °C. The reaction was brought to room temperature and stirred for 30 min. Then a solution of 3.54 g (14.8 mmol) of *N-t*-butyloxycarbonylethanolamine-*O*-methanesulfonate in 10 ml of dimethylformamide was added dropwise over 5 min. The reaction was heated to 70 °C for 3 h and then quenched by the addition of 25 ml of water. Ethyl acetate (100 ml) was added and the organic phase was separated and washed five times with 25 ml of water and once with 25 ml of saturated aqueous sodium chloride. The solution was dried over magnesium sulfate, filtered and concentrated in vacuo. The product was purified by flash chromatography on silica gel using 5% methanol in chloroform as eluent. The product (5.45 g, 71% of theoretical yield) was isolated as a solid. NMR ($CDCl_3$) 1.45 (9H, s), 1.5–1.6 (2H, m), 1.65–1.75 (2H, m), 1.8–1.9 (2H, m), 2.3–2.4 (2H, m), 2.5–2.8 (6H, m), 3.2–3.3 (2H, m), 3.7–3.8 (4H, m), 4.0–4.1 (2H, m), 4.6 (2H, s), 6.8–6.9 (3H, m), 6.9–7.1 (2H, m), 7.2–7.3 (3H, m), 7.4–7.5 (2H, m).

3-(2-Aminoethyl)-8-[3-(4-fluorobenzoyl)propyl]-4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one trihydrcchloride (AES) (Fig. 1, product 6). 3-(2-*N*-*t*-Butyloxycarbonylethyl)-8-[3-(2-4'-fluorophenyl-2-dioxolanyl)propyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (6.77 g, 11.6 mmol) was added to 20 ml of 4 M HCl in dioxane at room temperature. The reaction was stirred for 30 min at room temperature and then 20 ml of water was added. The reaction was stirred for an additional 1 h before all solvents were removed in vacuo. The resulting solid was triturated with diethyl ether and then recrystallized from ethanol/diethyl ether to give 5.43 g (86% of theoretical yield) of a white solid; m.p. 160–161°C. NMR ($CDCl_3$) 2.0–2.2 (4H, m), 3.0–3.2 (6H, m), 3.2–3.3 (2H, m), 3.5–3.8 (6H, m), 4.8 (2H, s), 6.8 (1H, t), 7.1–7.2 (2H, m), 7.2–7.3 (2H, m), 7.4 (2H, t), 8.05–8.1 (2H, m). Analysis ($C_{25}H_{31}N_4O_2F$·3HCl) C, H, N. MS (DCI/NH3) $M + H^+ = 439$.

Immobilization of AES on Sepharose 6B

AES was dissolved in DMF, brought to 45% DMF with buffer containing 0.3 M sodium carbonate, pH 11 and added to epoxy-activated Sepharose 6B at a ratio of 5.5 mg AES/ml resin. The mixture was incubated with shaking at 40°C for 12–16 h. The affinity matrix was washed extensively with DMF/water (45:55, v/v) and equilibrated into water. Unreacted groups were blocked with 2 M ethanolamine in buffer containing sodium bicarbonate (pH 9.5) for 18 h at 25°C. The resin was washed with water (20 vol.) and equilibrated into methanol/water (75:25, v/v) (10 vol.) and incubated with mixing overnight at 25°C. The resin was washed with methanol/water (20 vol.), reequilibrated into water and washed alternatively two times each (20 vol.) with buffer containing 0.1 M sodium acetate (pH 4.0) and 0.5 M NaCl, and buffer containing 0.3 M sodium bicarbonate (pH 8.2) and 0.5 M NaCl, followed by a single wash (20 vol.) with buffer containing 50 mM Tris-HCl (pH 7.5 at 25°C), 2 mM EDTA, 2 mM $MgCl_2$ (TEMg buffer) and 1.5 M NaCl and finally with TEMg buffer (5 vol.) containing 0.1 M NaCl. The resin was stored in TEMg buffer containing 100 mM NaCl and 0.01% NaN_3.

Membrane preparation

Bovine striata were homogenized with 20 vol. (w/v) TEMg buffer containing 250 mM sucrose and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at $1500 \times g$ for 15 min. and the resulting pellet was re-homogenized and centrifuged as before. The supernatants were combined and centrifuged at $28000 \times g$ for 20 min. The pellets were washed once with TEMg buffer containing 250 mM sucrose and 0.1 mM PMSF and once with TEMg buffer containing 0.1 M NaCl, 0.01% sodium ascorbate and 0.1 mM PMSF. Membranes were resuspended in 8% the original homogenate volume and stored at −80°C until use.

Photoaffinity labeling

Bovine striatal membranes were diluted to a final protein concentration of 0.2 mg/ml with 50 mM Tris-HCl (pH 7.5 at 25°C), 20 mM EDTA, 2 mM $MgCl_2$, 100 mM NaCl, 0.1 mM PMSF, 15 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor and 5 μg/ml leupeptin and incubated with 125 pM [^{125}I]N_3-NAPS in the presence or absence of varying concentrations of AES or 1 μM (+)-butaclamol. Membranes were exposed to ultraviolet light and processed as described [12,20]. Samples were resuspended in 62.5 mM Tris-HCl (pH 6.8) 10% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.003% Bromophenol blue, resolved on discontinuous 10% SDS-polyacrylamide gels [29] and subjected to autoradiography.

Detergent solubilization of membranes

Membranes were incubated with 0.25% sodium cholate in the presence of 1.5 M NaCl in TEMg buffer containing 0.1% sodium ascorbate, 0.1 mM PMSF, 15 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor and 5 μg/ml leupeptin at a final protein concentration of 5–6 mg/ml. The mixture was incubated with gentle shaking for 1 h followed by centrifugation at $100000 \times g$ for 1 h. The supernatant (cholate-solubilized receptor) was diluted with 1 vol. TEMg buffer and centrifuged at $100000 \times g$ for 1 h and the pellet resuspended and solubilized (approx. 1.4 mg protein/ml) using TEMg buffer containing 0.5% digitonin, 0.1 M NaCl and proteinase inhibitors described above. The mixture was centrifuged at $100000 \times g$ for 1 h, the supernatant (cholate-digitonin-solubilized receptor) was removed and used immediately or stored at −80°C.

AES-Sepharose adsorption studies

Cholate-digitonin-solubilized receptor was incubated with AES-Sepharose at various receptor to resin ratios with gentle shaking for 16 h at 4°C. The resin was separated from the supernatant by centrifugation at $1000 \times g$ for 5 min. Specific [^{3}H]spiperone-binding activity was assayed in the supernatant directly and after chromatography on Sephadex G-50 columns (25×0.75 cm) in TEMg buffer containing 0.1 M NaCl and 0.1% digitonin.

Chromatography on AES-sepharose

Cholate-digitonin-solubilized receptor was incubated with AES-Sepharose ((8–10):1, v/v) with gentle shaking for 16 h at 4°C. The mixture was centrifuged at $1000 \times g$ for 5 min, the supernatant was decanted, and the resin was washed twice by resuspension and centrifugation using 10 vol. TEMg containing 0.1 M NaCl and 0.1% digitonin. The washed resin was poured into a

column and washed with buffer (20 vol.) and brought to 22°C. The column was eluted with 50 μM haloperidol in TEMg containing 0.1 M NaCl, 0.1% digitonin and proteinase inhibitors. The column eluate was collected on ice. Samples were resolved on SDS-polyacrylamide gels or processed for binding assay as follows. Unbound haloperidol was removed by chromatography of column eluates (1 ml) on Sephadex G-50 (25 × 0.75 cm) equilibrated in TEMg containing 0.1 M NaCl, 0.1% digitonin and 1 mg/ml bovine serum albumin. Pooled samples (2.5 ml/column) were used for equilibrium-binding analysis or ligand competition studies.

Protein analysis

Proteins were determined by the method of Bradford [30] or by densitometric analysis of samples resolved on SDS-polyacrylamide gels and silver-stained [31] using crystalline bovine serum albumin as a standard. Eluates from the AES-Sepharose affinity matrix containing specific [^{3}H]spiperone-binding activity were pooled, lyophylized and resuspended in one tenth the original volume of SDS-polyacrylamide sample buffer (62.5 mM Tris-HCl (pH 6.8), 2.3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.003% bromophenol blue). Samples were resolved on 10% SDS-polyacrylamide gels along with

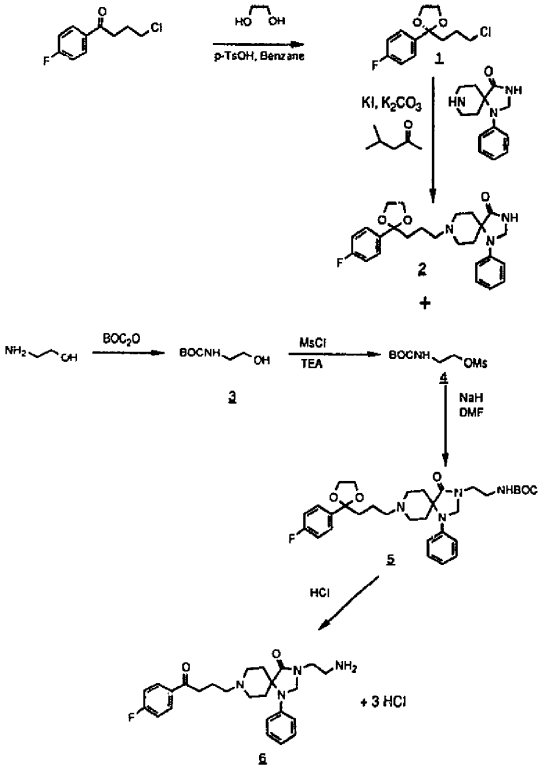


Fig. 1. Synthesis of AES.

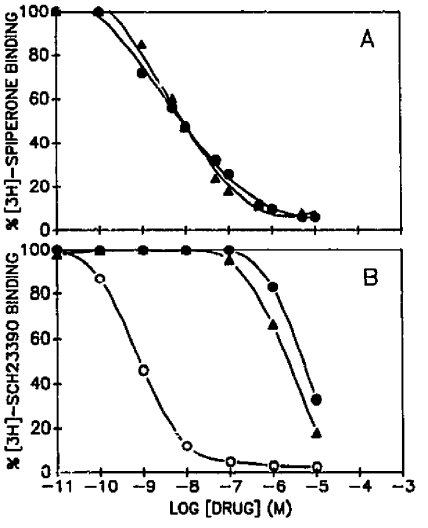


Fig. 2. Competition of AES for [3H]spiperone- (A) and [3H]SCH-23390- (B) binding sites in bovine striatal membranes. (A) Membranes (0.57 mg/ml protein) were incubated with 1 nM [3H]spiperone, the dopamine D2 antagonist, in the absence or presence of increasing concentrations of unlabeled spiperone (●) or AES (▲) for 18 h at 4°C. (B) Bovine striatal membranes were incubated with 1 nM [3H]SCH23390, the dopamine D1 antagonist, in the absence or presence of increasing concentrations of unlabeled SCH23390 (○), spiperone (●) or AES (▲). Membrane samples (0.5 ml) were washed four times with 4 ml TEMg buffer containing 100 mM NaCl, collected on glass-fiber filters and counted for radioactivity.

standards containing 0, 5, 10, 20, 40, 60, 80 and 100 ng bovine serum albumin. Quantitative analysis of silver-stained gels was performed using an LKB UltroScan XL laser densitometer.

Results

A butyrophenone molecule, AES, was synthesized for use in the preparation of an affinity chromatography matrix for dopamine D2 receptor purification (Fig. 1). The synthesis of AES was based on a modification of the NAPS synthesis [14]. The known 2-(3-chloropropyl)-2-(4-fluorophenyl)dioxolane [28] was condensed with commercially available 1-phenyl-1,3,8-triazaspiro[4.5]-decan-4-one in a catalytic Finkelstein reaction. The ketal-protecting group allowed selective amide anion formation with sodium hydride in dimethylformamide. *N-t*-Butyloxycarbonylaminoethyl-2-methanesulfonate was made by protection of the nitrogen with di-*t*-butyl-dicarbonate and mesylation of the alcohol. The amide anion cleanly alkylated at 70°C in DMF. The *N-t*-butyloxycarbonyl group was removed by anhydrous hydrogen chloride treatment. Then, dilution of the reaction mixture with water allowed the aqueous acid-catalyzed hydrolysis of the ketal. The final product was recrystallized as the trihydrochloride to give a 43%

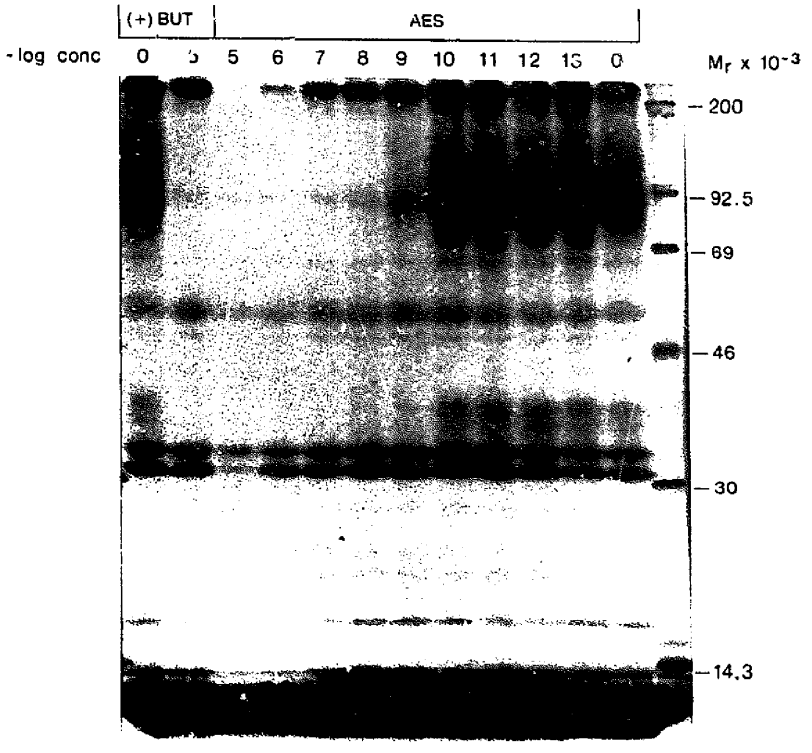
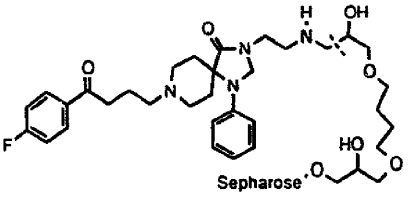


Fig. 3. Competition of AES for [^{125}I]N_3-NAPS photoaffinity labeling of bovine striatal membranes. Membranes were incubated in the absence or presence of increasing concentrations of AES or 1 μM (+)-butaclamol for 1 h at 25 °C. Ultraviolet irradiation was performed as described [12,20]. Samples were resolved on 10% SDS-polyacrylamide gels followed by autoradiography. Molecular weight markers (^{14}C-labeled) are shown on the far right: myosin (200000), phosphorylase *B* (92500), bovine serum albumin (69000), ovalbumin (46000), carbonic anhydrase (30000) and lysozyme (14300).

overall yield from commercially available materials. NMR and mass spectral analysis, as well as combustion analysis confirmed the product structure.

The pharmacological characteristics of AES were examined by competition for [^{3}H]spiperone and [^{3}H] SCH23390 binding to bovine striatal membranes. AES ($K_i \approx 1.7$ nM) showed similar affinity as the parent compound spiperone ($K_i \approx 0.8$ nM) in competing for [^{3}H]spiperone-binding sites ($K_d = 0.6$ nM) in membrane preparations (Fig. 2A). Like spiperone, AES showed no significant binding affinity ($K_i > 1$ μM) for the dopamine D1 receptor as assessed by competition for [^{3}H]SCH23390 binding to membrane preparations (Fig. 2B). The binding specificity of AES for the bovine striatal dopamine D2 receptor was further characterized by covalent photoaffinity labeling. Membranes were photoaffinity labeled with [^{125}I]N_3-NAPS in the absence and presence of varying concentrations of AES or in the presence of the dopaminergic antagonist, (+)-butaclamol, as a control. AES competes with high affinity for binding to a labeled peptide with $M_r \approx 90000$. This is the same labeled protein that is specifically displaced by (+)-butaclamol (Fig. 3).

AES was immobilized on epoxy-activated Sepharose 6B as described in Materials and Methods (Fig. 4). Coupling was performed in the presence of 45% DMF due to the limited solubility of AES in aqueous solution. Extensive washing of the resin after the coupling and



AES-Sepharose

Fig. 4. Preparation of AES-Sepharose 6B. AES was immobilized on epoxy-activated Sepharose as described in Materials and Methods.

blocking steps was necessary to eliminate non-specific trapping and subsequent leeching of free ligand by the affinity matrix. All batches of affinity resin were examined for the presence of free ligand prior to use in receptor chromatography studies. The soluble receptor-containing preparation was incubated with resin and specific [^{3}H]spiperone binding was assayed in supernatant fractions before and after chromatography on Sephadex G-50 columns. In this assay, preparations containing free AES mask [^{3}H]spiperone-binding sites in the supernatant fraction which are subsequently revealed after Sephadex G-50 chromatography and exchange of radioligand for unlabeled compound. Generally, there was no more than a 10–15% discrepancy between values obtained by direct binding and after chromatography on Sephadex G-50.

Dopamine D2 receptor was solubilized from bovine striatal membranes using a two-step detergent procedure. Membranes were treated first with 0.25% cholate in the presence of high ionic strength, followed by precipitation of soluble dopamine D2 receptor-binding activity and treatment with 0.5% digitonin. The average yield of specific [^{3}H]spiperone-binding activity in 15 separate experiments was 6% of the membrane bound activity. The pharmacological characteristics of the cholate and cholate-digitonin-solubilized preparations were examined by competition analysis. The rank order of potency for displacement of [^{3}H]spiperone binding by dopaminergic antagonists did not differ between bovine striatal membranes (Fig. 5A) and either the cholate- (Fig. 5B) or cholate-digitonin- (Fig. 5C) solubilized preparations. Displacement of [^{3}H]spiperone binding was stereospecific as demonstrated by the lack of displacement with (−)-butaclamol. No displacement of [^{3}H]spiperone-binding activity was seen with compounds that bind preferentially to the dopamine D1 receptor (SCH23390) or the β-adrenergic receptor (alprenolol, data not shown). Ketanserin, which binds to the serotonin 5HT2 receptor, displaced approx. 15% of the [^{3}H]spiperone-binding activity in membrane preparations, but did not compete for binding activity in either the cholate- or the cholate-digitonin-solubilized preparation.

Adsorption of [^{3}H]spiperone-binding activity by the affinity chromatography matrix was examined at different receptor to resin ratios (Fig. 6). Nearly 100% of the specific [^{3}H]spiperone binding activity was retained at a receptor to resin ratio of 2:1, whereas 60–70% of this activity was retained at a ratio of 10:1. Ratios of receptor to resin of (8–10):1 were chosen for chromato-

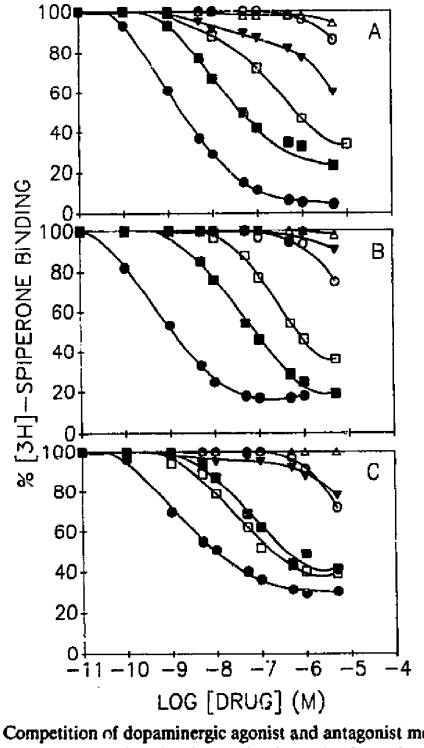


Fig. 5. Competition of dopaminergic agonist and antagonist molecules for [^{3}H]spiperone-binding sites in: (A) bovine striatal membranes, (B) cholate-solubilized and (C) cholate-digitonin-solubilized preparations. Receptor preparations were incubated with 1 nM [^{3}H]spiperone in the absence or presence of increasing concentrations of competing unlabeled ligand for 18 h at 4°C: spiperone (●), (+)-butaclamol (■), (−)-butaclamol (△), *N*-propylnorapomorphine (□), SCH23390 (○), ketanserin (▼). Membrane samples were processed as described in Fig. 2. Soluble receptor-containing samples (0.5 ml) were treated with 100–200 µl dextran-coated charcoal (1% activated charcoal, 0.1% dextran in TEMg buffer containing 100 mM NaCl) to remove unbound ligand.

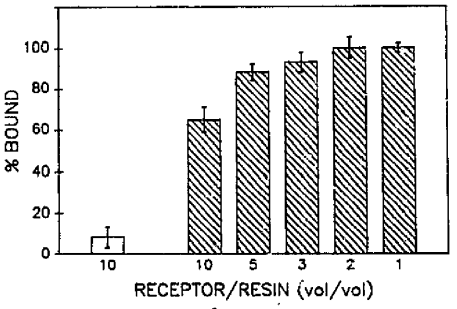


Fig. 6. Adsorption of specific [^{3}H]spiperone-binding activity by AES-Sepharose at varying receptor to resin ratios and to the matrix prepared in the absence of AES. Cholate-digitonin-solubilized receptor preparation (0.59 mg/ml protein) was incubated with resin prepared in the absence of AES (open bar) or with AES-Sepharose (hatched bars) for 18 h at 4°C at varying receptor to resin ratios. Adsorption of specific [^{3}H]spiperone-binding activity to the resin was calculated as the difference between the activity present in the starting cholate-digitonin-solubilized preparation (145 fmol/ml) and the activity remaining in the supernatant fraction. Data are expressed as the mean ± S.D. of triplicate determinations. Adsorption at the highest receptor to resin ratio (10:1) corresponds to 950 fmol specific [^{3}H]spiperone-binding activity per ml resin.

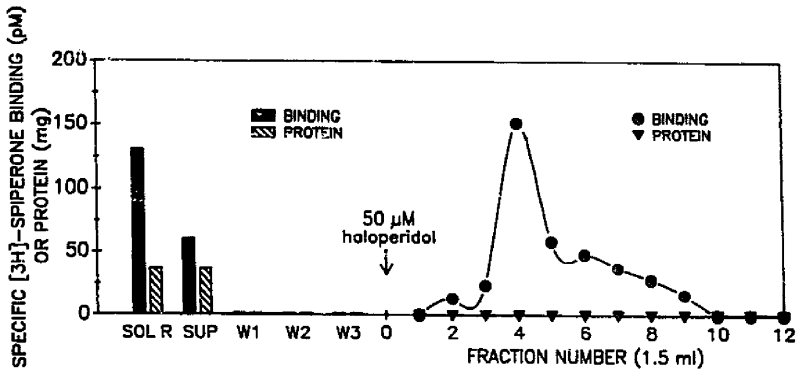


Fig. 7. Chromatography of cholate-digitonin-solubilized receptor on AES-Sepharose. In this experiment, 50 ml of cholate-digitonin-solubilized (0.7 mg/ml protein) bovine striatal membranes was incubated with 5 ml affinity matrix for 16 h at 4° C. Washing of the affinity resin and elution with 50 μM haloperidol was performed as described in Materials and Methods. Specific [^{3}H]spiperone-binding activity is shown in the solubilized preparation, resin supernatant and wash fractions (solid bars) and in the material eluted from the column (●). Protein was measured using the Bradford assay and is shown in solubilized preparation, resin supernatant and wash fractions (hatched bars). Protein was not measurable in eluate fractions (▼) using a modification of the Bradford assay that is sensitive to approx. 1 μg protein/ml. This column profile is representative of at least ten different experiments in which an average of 9% of the adsorbed [^{3}H]spiperone-binding activity was recovered in the column eluate.

graphic studies because the greatest absolute amount of binding activity was retained under these conditions. When the affinity matrix was prepared in the absence of AES, no significant [^{3}H]spiperone-binding activity was adsorbed.

Chromatography of cholate-digitonin-solubilized receptor on AES-Sepharose was performed and a representative chromatographic profile is shown in Fig. 7. Solubilized receptor preparation was incubated with affinity resin in batch form, the non-adsorbed material was removed by centrifugation, and the affinity matrix was washed as described in Materials and Methods and eluted with 50 μM haloperidol. Approx. 55% of the specific [^{3}H]spiperone binding in the solubilized preparation was adsorbed to the resin under these conditions. Nearly 100% of the protein in the solubilized preparation remained in supernatant fraction after batch adsorption of receptor to the affinity resin. Elution of the receptor from AES-Sepharose was measured by specific [^{3}H]spiperone binding of column fractions after removal of excess haloperidol by gel-filtration column chromatography. In the experiment shown in Fig. 7, 15% of the specific [^{3}H]spiperone-binding activity adsorbed to the resin was accounted for in the column eluate. There was no detectable protein in the column eluate as assessed by an assay sensive to protein concentrations as low as 1 μg/ml. In order to estimate the degree of purification achieved by AES-Sepharose affinity chromatography, larger volumes of cholate-digitonin-solubilized receptor preparation (approx. 125 ml) were applied to the AES-Sepharose affinity matrix (15 ml) and eluted as described in Materials and Methods. Dopamine D2 receptor-containing fractions were pooled, lyophilized and resolved on 10% SDS-polyacrylamide gels, followed by silver staining and quantitative densitometric analysis. Linear regression analysis of the densities obtained for protein standard curves (0–100 ng protein/sample) yielded a correlation coefficient of 0.97. The concentration of protein in samples of pooled AES-Sepharose affinity-purified receptor eluates was determined by summing the densities in each gel sample lane and comparing these densities to the protein standard curve. Two separate chromatographic experiments yielded column eluates with specific activities of 545 and 509 pmol/mg protein. From this, an estimated 2100-fold purification of dopamine D2 receptor from bovine striatal membranes was achieved.

Binding characteristics of the affinity-purified dopamine D2 receptor preparations are shown in Fig. 8. Competition for [^{3}H]spiperone binding in the affinity-purified receptor preparation was similar to that observed in cholate-digitonin-solubilized dopamine D2 receptor-containing preparations (Fig. 8A). Competition with the active and inactive enantiomers of butaclamol for [^{3}H]spiperone in the purified preparation showed the characteristic dopamine D2 receptor stereoselectivity; (−)butaclamol was ineffective in displacing [^{3}H]-spiperone-binding activity. Compounds specific for the dopamine D1 receptor (SCH23390), the serotonin 5-HT2 receptor (ketanserin) and the β-adrenergic receptor (alprenolol) were ineffective in displacing [^{3}H]spiperone-binding activity to affinity-purified preparations when tested at a concentration of 1 μM. The binding of [^{3}H]spiperone to the affinity-purified receptor was saturable and of high affinity (Fig. 8B) Scatchard analysis revealed a K_d of 0.2 nM in the affinity-purified preparations, which is similar to values obtained in bovine striatal membranes (0.6 nM), cholate-solubilized

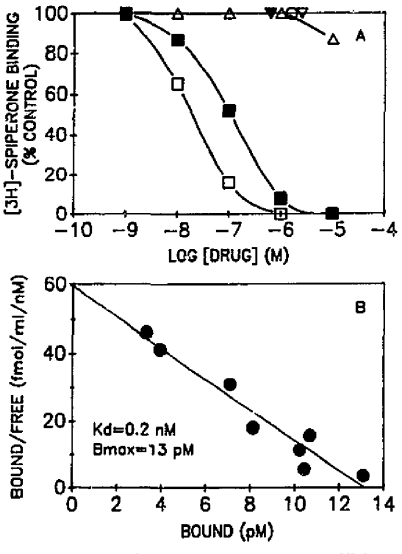


Fig. 8. Examination of the dopaminergic D2 specificity and equilibrium saturation binding analysis of affinity-purified receptor preparations. (A) Competition of dopaminergic and non-dopaminergic ligands for [^{3}H]spiperone binding to AES-Sepharose affinity column eluates. Column fractions (1 ml aliquots) were chromatographed on Sephadex G-50 columns to remove unbound haloperidol, and the eluate was incubated (0.5 ml) with 2 nM [^{3}H]spiperone in the absence or presence of varying concentrations of competing ligands: (+)-butaclamol (■), (−)-butaclamol (△), *N*-propylnorapomorphine (□); and a single concentration (1 μM) of: SCH23390 (○), ketanserin (▼) and alprenolol (▽). Bound [^{3}H]spiperone was separated from free compound using dextran-coated charcoal as described in Fig. 5. (B) Scatchard analysis of [^{3}H]spiperone binding to affinity-purified receptor preparation. Varying concentrations of [^{3}H]spiperone were incubated with AES-Sepharose column eluate in the absence or presence of 1 μM (+)-butaclamol.

receptor (0.5 nM) and cholate-digitonin-solubilized preparations (0.5 nM) (data not shown).

Discussion

This report describes the synthesis of a butyrophenone, AES, and use of this compound to prepare an affinity chromatography matrix for the purification of the dopamine D2 receptor. The receptor was solubilized from bovine striatal membranes and purified approx. 2100-fold by chromatography on AES-Sepharose 6B.

AES is a derivative of the high-affinity, specific dopamine D2 receptor antagonist molecule, spiperone. The affinity and specificity of AES for the dopamine D2 receptor was examined by competition for [^{3}H]spiperone- and [^{3}H]SCH23390-binding to dopamine D1 and D2 receptors in bovine striatal membranes. AES was equipotent with spiperone in competing for [^{3}H]spiperone-binding sites in membrane preparations. The molecule showed no significant binding to the dopamine D1 subclass of receptor, as assessed by competition for binding to [^{3}H]SCH23390, a selective dopamine D1 receptor antagonist ligand [1–3]. AES was also characterized by competition for membrane binding and covalent incorporation of [^{125}I]N_3-NAPS, a specific dopamine D2 receptor photoaffinity probe [12,14]. Covalent labeling of a protein with average $M_r \approx 90\,000$ in bovine striatal membranes was blocked by the inclusion of (+)-butaclamol (1 μM) and AES at concentrations in the nanomolar range. AES, like (+)-butaclamol, also competed for specific labeling of a smaller peptide of $M_r \approx 41\,000$. Interestingly, complete removal of N-linked oligosaccharide from the dopamine D2 receptor in rat [20] and dog [18,19] results in the formation of a peptide of $M_r \approx 40\,000$.

Solubilized dopamine D2 receptor was prepared by the sequential treatment of bovine striatal membranes with cholate in the presence of high ionic strength and with digitonin. The two-step procedure was necessary because solubilization with sodium cholate or digitonin alone yielded a receptor preparation that was unstable. Although specific [^{3}H]spiperone-binding activity in the cholate preparation appeared to adsorb to the AES affinity matrix, we were unable to elute receptor activity using elution procedures similar to those described for the cholate-digitonin-solubilized preparation. We found that the soluble dopamine D2 receptor activity in the cholate-solubilized preparation was precipitated by centrifugation if the concentration of detergent or salt was reduced. Because of the instability of this preparation, we collected the precipitate after solubilization of bovine striatal membranes with sodium cholate and treated the resuspended pellet with another detergent, digitonin (0.5%). This resulted in a solubilized receptor-containing preparation that was not unstable with changes in detergent concentration. Although we were able to prepare a stable soluble receptor preparation for affinity chromatography, this solubilization procedure yielded only 6% of the [^{3}H]spiperone-binding activity present in the starting membrane preparation. Omission of the sodium cholate solubilization step and direct solubilization with digitonin yielded preparations with greater variability in [^{3}H]spiperone-binding activity and required higher concentrations of digitonin (more than 1%) to achieve any significant degree of activity in the supernatant fraction.

When the cholate-digitonin-solubilized material was incubated with AES-Sepharose, [^{3}H]spiperone-binding activity was adsorbed and the efficiency of this process was dependent on the receptor to affinity matrix volume ratio. When a ratio of 2 vol. receptor to 1 vol. of resin was employed, nearly 100% of the [^{3}H]spiperone-binding activity was retained by the resin. Activity was not adsorbed if the affinity matrix was prepared in the absence of AES. Equilibrium saturation binding and ligand displacement of [^{3}H]spiperone binding of the AES-Sepharose affinity-purified receptor preparation

were characteristic of a dopamine D2 receptor. The binding was stereospecific with respect to the active and inactive enantiomers of butaclamol. The equilibrium dissociation constant obtained for [^{3}H]spiperone binding to the eluate (0.2 nM) is similar to that described in membrane and solubilized preparations. Reconstitution of affinity-purified receptor into phospholipid vesicles did not result in an increase in specific [^{3}H]spiperone-binding activity as has been described for purified dopamine D2 receptor preparations in other laboratories [22,25,27].

Several other laboratories have reported ligand affinity purification of the dopamine D2 receptor solubilized from a variety of tissues and species [21–27]. Previously described affinity matrices were all constructed using dopamine D2 receptor antagonists of the butyrophenone class of compounds. Resins have been prepared through the ketone in the 2 position of spiperone [22], as well as through the hydroxyl group in the 4′ position of the piperidine moiety of haloperidol [21,24,25,32] and the 4′ position of the aniline moiety of spiperone [23]. The resin described in this report is unique in that AES was efficiently linked to epoxy-activated Sepharose 6B through a stable aminoethyl moiety on the 3 position of spiperone.

Assuming a receptor $M_r \approx 90000$, we estimate a purification of 50000-fold would be required to bring the dopamine D2 receptor from bovine striatal membrane preparations (approx. 215 fmol/mg protein) to homogeneity. By this estimate, the AES-Sepharose affinity-purified receptor preparation described here is approx. 4% of homogeneity. This is similar to the purification reported by three other laboratories after ligand affinity purification of dopamine receptor solubilized from bovine striatum [21,25,26]. All three resins were constructed using haloperidol-related structures. With additional purification steps including molecular sizing and lectin affinity chromatography, two of these laboratories report purification of dopamine D2 receptor from bovine striatum to a specific activity of 2.5 nmol/mg protein (approx. 20000-fold) and show a major protein of approx. 95000 M_r after resolution on SDS-polyacrylamide gels [25,26]. Dopamine D2 receptor has also been purified from bovine anterior pituitary membranes to apparent homogeneity, revealing a protein with a distinctly different M_r (approx. 120000) than that described in bovine brain [27]. Resolution of highly purified receptor preparations in two dimensions should be useful in determining the purity of these preparations. It would also be of interest to compare amino-acid sequence information from pure preparations of dopamine D2 receptor to the amino-acid sequence deduced from cloning experiments [17]. Although numerous laboratories have developed procedures to affinity-purify the dopamine D2 receptor from a variety of sources, the quantity of material available from these efforts is very small. Cloning of the dopamine D2 receptor cDNA from rat brain should make the genetic overproduction of this receptor possible, perhaps even in soluble form. Affinity chromatography of receptor from such a source may facilitate the future biochemical characterization of larger quantities of this receptor.

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