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Affinity Chromatography of the Anterior Pituitary D_2-Dopamine Receptor†

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ABSTRACT: The D_2-dopamine receptor from bovine anterior pituitary has been solubilized with digitonin and purified ~1000-fold by affinity chromatography on a new affinity support. This support consists of a (carboxymethylene)oximino derivative of the D_2-selective antagonist spiperone (CMOS) covalently attached to Sepharose 4B through a long side chain. The interaction of the solubilized receptor activity with the affinity gel was biospecific. Dopaminergic drugs blocked adsorption of solubilized receptor activity to the CMOS–Sepharose with the appropriate D_2-dopaminergic potency and stereoselectivity. For agonists, (−)-*N*-*n*-propylnorapomorphine > 2-amino-6,7-dihydroxytetrahydronaphthalene ≃ apomorphine > dopamine, whereas for antagonists (+)-butaclamol >> (−)-butaclamol. The same D_2-dopaminergic specificity was observed for elution of receptor activity from the gel. To observe eluted receptor binding activity, reconstitution of the eluted material into phospholipid vesicles was necessary. Typically, 70–80% of the solubilized receptor was adsorbed by CMOS–Sepharose, and 40–50% of the adsorbed activity could be recovered after reconstitution of the eluted material. The overall recovery of D_2-receptor activity from bovine anterior pituitary membranes was 12–15% with specific binding activity of ~150 pmol/mg. The reconstituted affinity-purified receptor bound ligands with the expected D_2-dopaminergic specificity, stereoselectivity, and rank order of potency.

The D_2-dopamine receptor mediates many of the physiological actions of dopamine. It is found most prominently in the pituitary gland and the corpus striatum region of the brain [reviewed in Stoof & Kebabian (1984) and Seeman (1980)]. In the pituitary gland, the D_2 receptor mediates both the dopaminergic inhibition of prolactin release from the mammotrophs (Caron et al., 1978) and the inhibition of release of α-melanocyte-stimulating hormone from cells of the intermediate lobe (Cote et al., 1982), an effect which may be mediated through inhibition of adenylate cyclase activity (Giannattasio et al., 1981; Enjalbert & Bockaert, 1983). Recent evidence suggests that the D_2 receptor may also function via a different signal transfer pathway in the anterior pituitary (Canonico et al., 1982; Schofield, 1983). The ultimate understanding of the molecular events involved in the dopaminergic signal transduction which control hormone secretion will require the eventual purification of the receptor as well as the various other components of the signal transfer system (Sternweis et al., 1981; Bokoch et al., 1984; Codina et al., 1984; Pfeuffer et al., 1985).

Purification of the D_2-dopamine receptor has not yet been accomplished mainly due to the lack of suitable tools to this

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end. Many previous reports have appeared on the solubilization and characterization of functional D_2-dopamine receptors from pituitary and from corpus striatum [reviewed in Kilpatrick & Caron (1984), Hall et al. (1983), and Wouters et al., (1984)]. These reports have documented that the receptors can be solubilized with retention of their binding properties and interact with a guanine nucleotide regulatory protein (N) (Kuno et al., 1983a; Kilpatrick & Caron, 1983), possibly the inhibitory regulatory N_i protein (Cronin et al., 1983; Kuno et al., 1983b; Cote et al., 1984). Previous attempts at purification of the D_2-dopamine receptor using gel filtration (Lilly et al., 1981) or lectin chromatography (Lew & Goldstein, 1984) have yielded only a fewfold enrichment of the activity.

In this paper, we report the development of a new affinity chromatography matrix for the biospecific purification of the D_2-dopamine receptor. This affinity matrix yields an ~1000-fold-purified receptor preparation from bovine anterior pituitary membranes which retains its pharmacological specificity for binding dopaminergic ligands.

Materials and Methods

Materials

Spiperone was a gift from Janssen Pharmaceuticals (Beerse, Belgium). Frozen bovine anterior pituitaries were purchased from JR Scientific (Woodland, CA). Other chemicals and drugs used were from sources previously described (De Lean et al., 1982; Cerione et al., 1983).

Methods

Synthesis of CMOS. [(Carboxymethylene)oximino]spiperone (CMOS)[1] was synthesized by refluxing carboxymethoxylamine hemihydrochloride (0.98 g, 4.5 mmol) with spiperone (1.78 g, 4.5 mmol) in 20 mL of ethanol for 4 h. The mixture was evaporated to dryness, and the product was purified by flash chromatography on silica gel eluting with 5% methanol/methylene chloride to give 1.89 g of product (90%): NMR (Me_2SO-d_6) δ 1.6–2.1 (4 H, multiplet, aliphatic), 2.7–3.3 (10 H, multiplet, aliphatic), 4.7 (4 H, doublet, –O–CH_2–CO_2H, N–CH_2–NH–), 6.8–7.1 (3 H, multiplet, aromatic), 7.2–7.5 (4 H, multiplet, aromatic), 7.8–8.0 (2 H, multiplet, aromatic), 9.95 (1 H, singlet, –COOH, exchangeable with D_2O). Anal. Calcd for $C_{25}H_{29}N_4O_4F \cdot {}^1/_2H_2O$: C, 62.35; H, 6.31; N, 11.29. Found: C, 62.89; H, 6.28; N, 11.74.

Immobilization of CMOS. Epoxy-Sepharose 4B was prepared as previously described (Caron et al., 1979) and converted to a free amino-containing Sepharose as follows: 50 mL of packed epoxy-Sepharose 4B was added to 100 mL of 1 M ethylenediamine in 0.1 M Na_2CO_3, pH 10.0, at 22 °C for 16–18 h. The gel was then washed with 10 volumes of distilled water, 0.2 M acetic acid, and 50 mM NaOH and water again until the pH of the effluent was ~5. CMOS was dissolved in Me_2SO (100 mg in 50 mL), and the amino-Sepharose 4B (50 mL equilibrated in H_2O) was added slowly to the CMOS solution (pH 4.5). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) (1 g/50 mL of gel) was added, and the pH was adjusted again to 4.5. The mixture was reacted for 12–16 h at 22 °C after which 1 g of EDAC was added again and reacted for another 8–10 h. The derivatized gel was washed with 50% Me_2SO/50% water (0.5 L/50 mL) and distilled water (2.5 L/50 mL) over a scintered glass funnel and stored at 4 °C with 0.02% NaN_3. A similar derivative of spiperone has been previously conjugated to bovine serum albumin in an attempt to raise antibodies to spiperone (Schreiber et al., 1983).

Membrane Preparation and Receptor Solubilization. Bovine pituitary membranes and soluble preparations were obtained as described previously (Kilpatrick & Caron, 1983; De Lean et al., 1982) with the addition of 10 μg/mL leupeptin, 0.1 mM PMSF, and 2 mM EDTA during homogenization and solubilization.

Affinity Chromatography of the Solubilized Receptor. The CMOS–Sepharose was washed with 2–4 bed volumes of 50 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% digitonin, pH 7.4. Soluble preparations were loaded batchwise by incubation overnight at 4 °C with the CMOS–Sepharose. Typically, 2 mL of soluble preparation was loaded/mL of CMOS–Sepharose. The gel was then washed in a column at 4 °C with 10–15 bed volumes of buffer consisting of 50 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, and 0.1% digitonin, pH 7.4 (flow rate 200 mL/h for 2–3 h). For elution, the column was brought to 22 °C and eluted with 2 bed volumes of 10 μM haloperidol in the wash buffer at a flow rate of 50 mL/h. The eluted fractions were collected on ice and desalted by Sephadex G-50 chromatography (Caron et al., 1979).

Reconstitution of D_2-Receptor Activity. Reconstitution of D_2-receptor activity was performed as previously described for the β-adrenergic receptor except that the ultracentrifugation step was omitted (Cerione et al., 1983).

Receptor Binding Assay. All radioligand binding assays for membranes and detergent-solubilized preparations were performed as described previously (Kilpatrick & Caron, 1983; De Lean et al., 1982). Reconstituted receptor activity was also assayed by Sephadex G-50 chromatography.

Protein Determination. Protein was determined by Bradford (Bradford, 1976) and by amidoschwartz (Schaffner & Weissman, 1973) protein assays with bovine serum albumin as standard.

NMR Spectroscopy. Spectra were obtained with a Varian XL-300 operating at 300 MHz for ^1H internally referenced with tetramethylsilane.

Results

Chromatography of Bovine Anterior Pituitary Membrane Solubilized Preparations on CMOS–Sepharose. [(Carboxymethylene)oximino]spiperone which displays a dissociation constant of 150 nM for the receptor in ligand binding assays (data not shown) was immobilized on activated Sepharose 4B via an amide linkage using water-soluble EDAC. The structure of the affinity chromatography matrix is shown in Figure 1 (inset). A chromatographic profile of digitonin-solubilized receptor on CMOS–Sepharose is shown in Figure 1. Approximately 70–80% of the [^3H]spiperone binding activity adsorbed to the CMOS–Sepharose while most of the protein in the soluble preparation was unretarded.

Elution of the CMOS–Sepharose as measured directly by [^3H]spiperone resulted in low yields of receptor activity (<5%). One possible reason for the apparent low recovery was that the purified D_2 activity was extremely labile. Attempts to stabilize the receptor during and after elution by including stabilizing agents (DTT, glycerol, BSA, phospholipids, etc.), by replacing digitonin with various other detergents, by adding additional protease inhibitors, or by eluting with various other

[1] Abbreviations: CMOS, [(carboxymethylene)oximino]spiperone; NPA, (–)-*N*-*n*-propylnorapomorphine; APO, apomorphine; ADTN, 2-amino-6,7-dihydroxytetrahydronaphthalene; DA, dopamine; NAPS, *N*-(*p*-aminophenethyl)spiperone; EDAC, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; Me_2SO, dimethyl sulfoxide; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; BSA, bovine serum albumin.

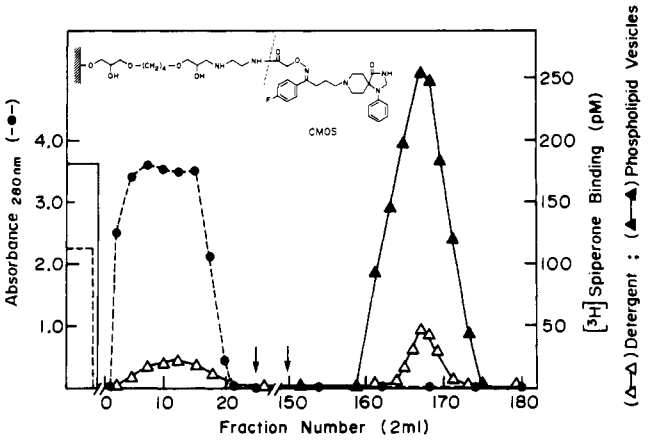

FIGURE 1: Chromatography of digitonin-solubilized D_2-dopamine receptor on CMOS–Sepharose. In the experiment shown, 70 mL of digitonin-solubilized receptor was incubated and chromatographed on a 40-mL CMOS–Sepharose column (1.5 × 30 cm). Chromatography and the assays were performed as described under Methods. Note that the assessment of receptor activity in solubilized preparations (such as pass-through material) is not dramatically underestimated as it is by direct [^{3}H]spiperone binding to eluted material. Indeed, solubilized preparations show only a slight increase in activity (1.5–2.0-fold) upon reconstitution. The bars at the left of the figure represent respectively the absorbance (280 nm) (---) and receptor binding activity (—) present in the soluble preparations. The arrow at fraction 23 indicates the start of the wash procedure whereas the arrow at fraction 150 shows the start of the elution by the addition of buffer containing 10 μM haloperidol. Inset: Structure of CMOS–Sepharose 4B. These results are representative of at least five different experiments.

selective agonists and antagonists were all unsuccessful. However, reinsertion of the receptor activity into phospholipid vesicles yielded a 5–10-fold increase in the recovery of [^{3}H]spiperone binding activity (Figure 1). Routinely, the recovery of [^{3}H]spiperone binding sites from the CMOS–Sepharose after reconstitution into phospholipid vesicles was 40–50% of the sites originally adsorbed to the affinity gel. Thus, it appears that the D_2-receptor binding activity requires a lipid milieu to bind ligand after chromatography on the CMOS affinity gel.

Specificity of Interaction of Solubilized D_2 Receptors with CMOS–Sepharose. As shown in Figure 2A, receptor adsorption to CMOS–Sepharose could be blocked by prior exposure of the receptor to a variety of dopaminergic ligands. Agonists (100 μM) and antagonists (10 μM) blocked adsorption of receptor activity to the CMOS–Sepharose with the expected order of efficacy: NPA > ADTN $\simeq$ APO > DA; spiperone $\simeq$ (+)-butaclamol > haloperidol $\simeq$ sulpiride >> (−)-butaclamol. The spirodecanone site blocker R5260 was ineffective as was the S_2-serotonin-selective antagonist ketanserin.

The specificity of elution from CMOS–Sepharose was also investigated (Figure 2B). The eluted receptor activity was assayed after reconstitution into phospholipid vesicles. The specificity of elution was essentially the same as that for inhibition of adsorption. Note that these interactions displayed the expected stereoselectivity of (+)-butaclamol vs. (−)-butaclamol. SCH 23390 (a D_1-dopamine-selective antagonist), alprenolol and epinephrine (β-adrenergic agents), and ketanserin, (a serotonin antagonist) were ineffective (data not shown). The combined results from Figure 2A,B suggest that the D_2-dopamine receptor interactions with CMOS–Sepharose are biospecific.

Characteristics of the Affinity-Purified D_2-Dopamine Receptor Preparations. Typically 300 mL of bovine anterior pituitary membranes (3.6 mg/mL, 168 pmol of [^{3}H]spiperone

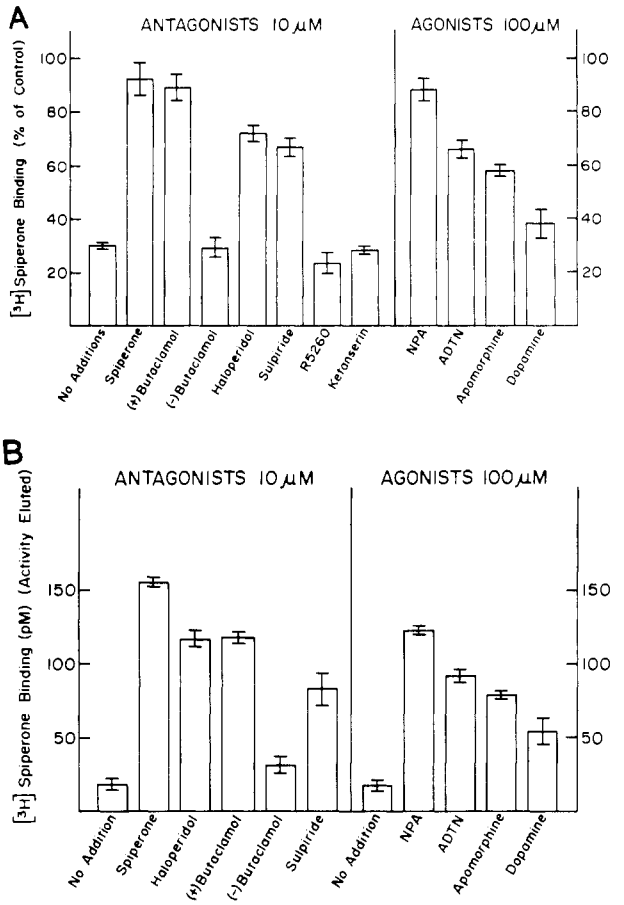

FIGURE 2: (A) Specificity of adsorption of solubilized D_2-dopamine receptor activity to CMOS–Sepharose. Three milliliters of solubilized receptor preparations was preincubated with the indicated drugs for 20 min at 25 °C and chromatographed through 1 mL of CMOS–Sepharose. The effluent was desalted on Sephadex G-50 and assayed for [^{3}H]spiperone binding as described. The activities are expressed as the percent of control, in which the solubilized preparation was chromatographed on a 1-mL column of unsubstituted epoxy-Sepharose. Parallel experiments were also performed to correct for the incomplete removal of some agents by desalting. The data shown are representative of two such experiments. (B) Specificity of elution of receptor activity from CMOS–Sepharose. Three-milliliter aliquots of solubilized receptor (350 fmol) were incubated with 1-mL aliquots of CMOS–Sepharose for 4 h at 4 °C, poured into Pasteur pipets, and washed with 3 × 5 mL of 0.1% digitonin, 50 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, and 1 mM EDTA, pH 7.4 at 4 °C. The columns were eluted at 25 °C with 2 mL of the indicated drugs in wash buffer over a period of 30 min. The eluates were desalted, reconstituted, and assayed as described for [^{3}H]spiperone binding activity. Results are typical of two experiments.

binding, 0.155 pmol/mg) yields 100 mL of solubilized preparation (3.45 mg/mL, 45 pmol of [^{3}H]spiperone binding, 0.130 pmol/mg). Chromatography of the solubilized preparation on the affinity gel yields 10 mL of affinity-purified receptor [11.6 μg/mL, 20.8 pmol of [^{3}H]spiperone binding, 115–180 pmol/mg (n = 5)]. This results in an overall 750–1200-fold purification and 12–15% recovery of receptor activity. The recovery and specific activities were calculated by assuming complete reconstitution of activity and thus are probably underestimated. These results indicate that a substantial purification of D_2-dopamine receptor activity is obtained by one affinity chromatography step.

Figure 3 shows the binding specificity of the reconstituted affinity-purified material. The rank order of potency for agonists in competing for [^{3}H]spiperone binding is classically D_2-dopaminergic: NPA > ADTN > DA. The antagonist (+)-butaclamol is ~3 orders of magnitude more potent than

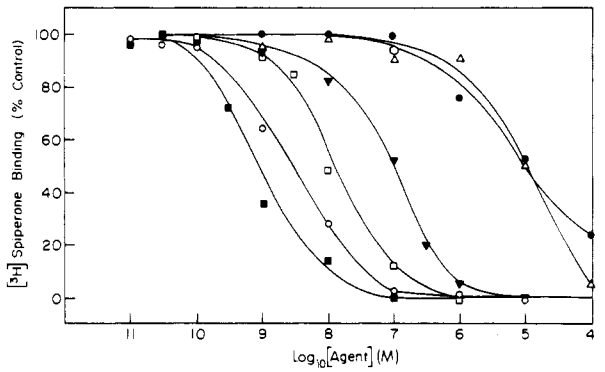

FIGURE 3: Specificity of binding of D_2-dopaminergic ligands to reconstituted affinity-purified receptor preparations. Increasing concentrations of dopaminergic ligands were incubated with 3–4 nM [^{3}H]spiperone in 0.1% ascorbate, 0.1 mM Gpp(NH)p, 50 mM Tris-HCl, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, pH 7.4, and reconstituted receptor preparations for 18 h at 4 °C. (■) Spiperone; (○) (+)-butaclamol; (□) NPA; (▼) ADTN; (●) dopamine; (△) (−)-butaclamol; (○) ketanserin. The bound ligand was separated by Sephadex G-50 chromatography. The 100% [^{3}H]spiperone bound in the figure corresponds to 150–250 pM. The data shown are representative of two to three determinations for each curve. The binding curves were analyzed as described previously (De Lean et al., 1982).

(−)-butaclamol in ligand competition assays. Spiperone, the D_2-selective antagonist, is potent whereas ketanserin, the potent serotonergic antagonist, does not compete for binding. These results demonstrate that the reconstituted affinity-purified receptor retains the D_2 pharmacological selectivity and specificity of the native receptor in the membrane.

Agonist displacement curves of the affinity-purified receptor preparations usually displayed biphasic competition curves in the absence of guanine nucleotides (data not shown). Addition of Gpp(NH)p converted these curves to monophasic, suggesting that the purified receptor preparations contain endogenous N proteins. The data shown in Figure 3 were obtained by using 0.1 mM Gpp(NH)p in the binding assays.

Table I compares the binding characteristics of the digitonin-solubilized receptor preparations and the reconstituted affinity-purified receptor preparations. It is evident that the stereoselectivity and specificity for ligands was maintained through the affinity chromatography step. These results show that the receptor activity, eluted from CMOS–Sepharose and reconstituted retains the pharmacological specificity expected of the D_2-dopamine receptor.

Discussion

The purification of the D_2-dopamine receptor has been hampered by the lack of a suitable affinity chromatography matrix. We report here the partial purification of the D_2-dopamine receptor from bovine anterior pituitary membrane by biospecific affinity chromatography on a newly synthesized Sepharose matrix which contains an immobilized analogue of the potent selective antagonist spiperone, [(carboxymethyleneoximino]spiperone. We have obtained 750–1200-fold purification by use of a single affinity chromatography step with an overall recovery of 12–15% calculated from bovine anterior pituitary membranes. The specific activities of the eluted material range from 115 to 180 pmol of [^{3}H]spiperone binding/mg of protein (n = 5). These specific activities are calculated from protein determinations (by the amidoschwartz method) of the eluates and from the [^{3}H]spiperone binding activity of the eluted material after reinsertion into phospholipid vesicles. Since the affinity-purified receptor in detergent has a diminished ability to bind ligands, we are unable to assess the true efficiency of reconsitution. While an efficiency of 100% reconstitution was assumed, it is likely to be substantially less (Cerione et al., 1983). This would result in an underestimation of the true specific activity and recovery of the receptor.

Table I: Comparison of K_D Values of Several Dopaminergic Agents for Binding to Solubilized and Affinity-Purified Reconstituted D_2-Receptor Preparations[a]

ligand	soluble K_D (nM)	affinity-purified reconstituted K_D (nM)
agonists		
NPA	24	10.9 ± 2.6
ADTN	51	32 ± 5.7
apomorphine	568	390 ± 2
dopamine	7300	976 ± 106
antagonists		
spiperone	0.47	0.82 ± 0.18
(+)-butaclamol	1.1	1.17 ± 0.40
(−)-butaclamol	1080	831 ± 230

[a] The K_D values for ligand binding to the soluble preparations were calculated from competition curves as described under Methods and according to De Lean et al. (1982). The K_D values for the affinity-purified reconstituted preparations are the mean ± SD of two to five K_D determinations, each curve being performed in triplicate.

Previous reports have alluded to a role of phospholipids in improving D_2-receptor activity in solubilized preparations (Wheatley et al., 1984; Wheatley & Strange, 1984). The requirement for reinsertion of the affinity-purified receptor into phospholipid vesicles in order to observe ligand binding could be due to one of several reasons. Reconstitution of the receptor may be necessary to assemble subunits into a binding configuration, as with the IgE receptor (Rivnay et al., 1984), or to confer to the receptor an appropriate tertiary structure. Disruption of tertiary or quaternary structure during the chromatography by stripping of a tightly bound phospholipid necessary for functional binding is also possible. It is evident from our data that some molecular changes occur upon purification which make reconstitution a necessary prerequisite for functional D_2-receptor binding.

We have observed in affinity chromatography purified preparations the reconstitution of a high-affinity agonist site, which is responsive to the presence of guanyl nucleotides. These results suggest the copurification of an N protein with the D_2-receptor activity. This finding is both intriguing and exciting as it opens the way for study of the receptor with its various effectors. Obviously, further work will be required on this system to elucidate the nature of the interactions of the receptor with these putative N proteins and the identities of the proteins themselves.

The affinity chromatography procedure described here should prove to be a key tool in the eventual purification of the D_2-dopamine receptor. The process by which purification is obtained appears to be biospecific since the interactions of the receptor with the affinity matrix display appropriate specificity. Approximately 1000-fold purification of the receptor is obtained in this single step with 40–50% of the solubilized receptor recovered. On the basis of the assumption that the ligand binding subunit of the receptor resides on a peptide of M_r 94 000 (Amlaiky & Caron, 1985), an additional 50–100-fold purification should be sufficient to yield homogeneous preparations of the receptor. Other procedures such as lectin chromatography and molecular sieve on high-performance liquid chromatography matrices coupled with this affinity chromatography procedure should represent a good strategy toward the goal of obtaining pure receptor preparations.

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Registry No. Carboxymethoxylamine hemihydrochloride, 2921-14-4; spiperone, 749-02-0; amino-Sepharose 4B, 74315-68-7; CMOS-Sepharose 4B, 100019-61-2; epoxy-Sepharose 4B, 7398-98-4; ethylenediamine, 107-15-3.

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