Purification and reconstitution of serotonin receptors from bovine brain

(affinity chromatography)

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Communicated by Kenneth V. Thimann, December 21, 1987

ABSTRACT An affinity-chromatography column was used to isolate and purify 5-hydroxytryptamine (serotonin, 5-HT) receptors from bovine brain frontal cortex. The affinity ligand lysergic acid ethylamidoethylbromide was synthesized and coupled to an agarose matrix via a thioether bond. Receptors in the crude membrane fragments were solubilized using 3-[3-(cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), affinity purified, and reconstituted into lipid vesicles. [3H]5-HT binding analysis indicates a single class of high-affinity binding site (Kd, 16.9 nM) that was reconstituted. 5-Methoxytryptamine, a competitor for high-affinity serotonin sites, inhibited this binding and showed a KI of 27.4 nM. Ketanserin, a high-affinity ligand for 5-HT2 type receptors, was ineffective in displacing [3H]5-HT binding at concentrations up to 4 μM indicating a 5-HT1 receptor as the primary receptor type isolated. The average specific activity of 359 pmol/mg in the reconstituted fractions is an enrichment of 1062-fold over crude membrane fragments. Sodium dodecyl sulfate electrophoresis showed the presence of four proteins in the reconstituted vesicles with approximate relative Mr, values of 63,000, 70,000, 81,000, and 94,000.

5-Hydroxytryptamine (serotonin, 5-HT) is a monoamine neurotransmitter in the central nervous system. Two studies have presented evidence that serotonin receptor dysfunction may be directly involved in mental disease. Mehli et al. (1) discovered lysergic acid diethylamide (LSD)-displacing factors in the cerebral spinal fluid of unmedicated psychotic patients. These factors competed with [3H]LSD for binding to serotonin receptors and were not present in the nonpsychotic control group. Todd and Ciaranello (2) have identified antibodies in the cerebrospinal fluid of an autistic child against human serotonin receptors. These studies indicate that some mental diseases may have an autoimmune etiology similar to that of the neuromuscular disease myasthenia gravis. Detailed studies of the receptor and its effector system have so far been difficult due to the small amounts of receptors expressed in brain tissue in terms of total neural protein.

Serotonin regulates two second-messenger systems involving adenylate cyclase activity (3–6) and phosphoinositol metabolism (7, 8). Pharmacologically the receptors are divided into two main classes—5-HT1 and 5-HT2 receptors (9, 10). 5-HT1 receptors are further divided into subtypes based on differing competition profiles for serotoninergic ligands (11–13). 5-HT1 receptors are modulated by guanine nucleotides (14, 15) and therefore are believed to be involved in adenylate cyclase regulation, whereas 5-HT2 receptors stimulate phosphoinositol turnover (7, 8). Studies in the mouse neuroblastoma fetal Chinese hamster cell hybrid line NCB-20 have shown that these cells contain two distinct serotonin receptors. One mediates adenylate cyclase (16, 17), and the other stimulates acetylcholine release (16). The regulation of adenylate cyclase by serotonin in this cell line is affected at a separate site by [D-Ala2,D-Leu5]enkephalin (18), a peptide transmitter. An intricate regulatory network must thus be involved in mediating cellular events by receptors at the cell surface. Systems have yet to be developed to study the molecular mechanisms of these neural networks.

Affinity chromatography has been used to purify the muscarinic acetylcholine receptor (19) and β-adrenergic receptor (20), which has made possible further studies on the structure, function, and molecular properties of these membrane proteins (21, 22). We report an affinity-chromatography column using a covalently bound LSD molecule as the affinity ligand (Fig. 1) to isolate and purify high-affinity serotonin receptors from bovine brain. The purified receptors were subsequently reconstituted in artificial membranes and characterized by pharmacological and electrophoretic methods.

MATERIALS AND METHODS

Sources. [3H]5-HT (24.4 or 26 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Serotonin (5-HT), 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS), D-lysergic acid, pargyline, and 5-methoxytryptamine were from Sigma. Ketanserin tartrate was from Jansen Pharmaceuticals (Belgium). 2-Ethylaminoethylbromide hydrobromide was from K & K. Affi-Gel 401 was from Bio-Rad, polyethylene glycol (Mr, 16,000–20,000) was from Aldrich, and asolecin was from Associated Concentrates (Woodside, NY). Centricon concentrators were from Amicon. Bovine brains were obtained at a local slaughterhouse.

Synthesis of Affinity Ligand. D,L-Lysergic acid ethylamidoethylbromide (LAEB) was synthesized by the mixed anhydride reaction for synthesis of lysergic acid amides developed by Pioch (23). No attempt was made to separate the isomers produced. Mass spectroscopy (Finnigan model 3200 GC/MS) of the free base and the tartrate salt of the ligand gave Mr, values of 404 and 475 compared with theoretical values of 402.3 and 477, respectively. The free base LAEB was stored at −20°C in 1-propanol before the coupling reaction with the agarose sulfhydryl gel.

Preparation and Solubilization of Crude Cortical Membrane Fragments. Frontal cortex was dissected with care to minimize white matter content. Tissue was homogenized as a 10% (wt/vol) solution in 0.32 M sucrose/2.5 mM Tris, pH 7.4. This homogenate was then centrifuged at 600 × g for 10 min. The pellet was discarded, and the supernatant was centrifuged at 50,000 × g for 25 min. The supernatant was discarded. To the pellet was added 2–3 pellet volumes of the

Abbreviations: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); LAEB, lysergic acid ethylamidoethylbromide; LSD, lysergic acid diethylamide; 5-HT, serotonin, 5-hydroxytryptamine; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate.

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sucrose solution; this solution was diluted 10- to 20-fold with distilled water, resuspended, and centrifuged at 50,000 × g for 25 min. This procedure was repeated three more times, the last two times without adding sucrose. The final pellet was resuspended in 50 mM Tris, pH 7.4, and is designated crude membrane fragments. To solubilize the receptor, crude membrane fragments were centrifuged at 50,000 × g for 30 min and resuspended in 1.25% (wt/vol) CHAPS/50 mM Tris, pH 7.4. Membrane fragments were resuspended in the CHAPS/Tris solution at proportions ranging from 3.3 mg of membrane protein per ml of CHAPS/Tris solution to 40 mg per ml of CHAPS/Tris solution. The overall protein yields ranged from 15% for the higher protein/CHAPS ratios to 20% for the lower ratios. The specific activities (as determined by [3H]5-HT binding assays) of solubilized fractions were independent of the solubilization yield. Consequently, most of our solubilization procedures used the higher protein/CHAPS ratios. The membrane fragment/CHAPS mixture was incubated with stirring on ice for 1½ hr and was then centrifuged at 100,000 × g for 60 min. The supernatant containing the solubilized fraction was saved.

**[3H]5-HT Binding Assays.** Two-tenths milliliter of membrane preparation was incubated in a total volume of 0.6 ml with appropriate radioligand concentration for 20 min at room temperature. Binding buffer consisted of 50 mM Tris, pH 7.4/6.56 μM CaCl2/1 mM ascorbic acid/20 μM pargyline. Nonspecific binding was defined by similar mixtures containing 500 μM 5-HT. The reaction was terminated by filtration through Whatman GF/B glass fiber filters under reduced pressure. The filters were then rinsed three times with 5 ml of ice-cold 2.5 mM Tris, pH 7.4. Bound radioactivity was counted by liquid scintillation. In experiments where solubilized samples were used, the mixture was first incubated with 25% PEG 1200 before filtering.

**Affinity Chromatography.** Approximately 25 ml of AffiGel 401 was exposed to diethiothreitol to reduce disulfides by incubating the gel in 25 ml of 20 mM diethiothreitol at 200 mM Tris, pH 8.0, for 30 min at room temperature. Dithiothreitol was removed from the column by washing with 50 mM Tris, pH 7.4, until the eluate was free of sulphydryl groups by 5',5'-dithiobis(2-nitrobenzoic acid) (DTNB) assays (50 μl of eluate was added to 1 ml of 0.323 mM DTNB/100 mM Tris, pH 8; presence of free sulphydryls causes yellow color). A 50-μl aliquot of the column material was also assayed with DTNB; a bright yellow color in the assay solution indicated the presence of available sulphydryl groups. The column was then equilibrated to 100% isopropanol by washing with three column volumes each of 25, 50, and 100% isopropanol solutions. The column was then resuspended and mixed with LAEB at a mole LAEB-to-sulphydryl group ratio of 50:1 in the agarose gel. The mixture was incubated overnight at room temperature. Excess ligand and bromine salts were washed out as the column was reequilibrated to aqueous environment in 50 mM Tris, pH 7.4. A 50-μl aliquot of the gel matrix was tested for sulphydryl group by the DTNB assay. A faint yellow color in the assay solution indicates a large proportion of the sulphydryls reacted with the affinity ligand. The resuspended column material was treated with 0.25 g of iodoacetamide at room temperature for 20 min. The iodoacetamide solution was removed by washing with 50 mM Tris, pH 7.4. The DTNB assay of the column material showed no difference from the distilled water control, indicating all remaining sulphydryls had been alkylated. Solubilized extract was loaded onto the column at a flow rate of 50 ml/hr and eluted with 2 mM CHAPS/50 mM Tris, pH 7.4, until protein concentration reached base level. The column was then specifically eluted with 120 μM 5-HT/4 mM CHAPS/800 μM ascorbic acid/5.3 mM CaCl2/16 μM pargyline containing asolectin at 3 mg/ml. Specifically eluted fractions were dialyzed against 50 mM Tris, pH 7.4, for reconstitution into receptor-containing lipid vesicles.

The percent of receptors recovered is calculated by dividing moles of receptor in the eluted fractions by total moles of receptor loaded on the column. Total moles of receptors loaded is defined by the specific activity (determined by [3H]5-HT binding assay at 50 nM) of the solubilized preparation multiplied by milligrams of protein loaded on the column. Moles of receptor in the eluted fractions can be determined from the area under the curve (see Fig. 2).

**Protein Concentration.** Protein concentration was determined by the method of Lowry et al. (24). Absorbance due to buffer and detergent was subtracted.

**NaDodSO4/PAGE.** Specifically eluted fractions were pooled and concentrated by reverse dialysis against 10% PEG (M, 16,000–20,000) followed by concentration using Centricon concentrators. For electrophoresis a 3.75% stacking gel and a 10% running gel were used. Samples were run under nonreducing conditions. The gel was visualized by silver staining (25).

## RESULTS

**Affinity Chromatography of Solubilized Bovine Cortical Membrane Fragments.** The solubilized membrane prepara-
tion was shown to have retained the \(^{3}H\)-5-HT binding activity by PEG precipitation before filtration. (Subsequent work using polyethyleneimine-treated filters and assessments of the detergent-solubilized receptors by their ligand-binding properties will be presented in a later publication.) Retention of the binding activity by detergent-solubilized receptors is necessary for the affinity-purification technique. This result also suggests that the functional structure of the receptor complex is not seriously disrupted. CHAPS-solubilized membrane fragments were run through the LSD affinity column, and \(^{3}H\)-5-HT binding activity was recovered over two ranges (Fig. 2). The first range recovered activity eluted with buffer, and its peak activity matched the peak protein concentration that passed through the column. This activity represents receptors that did not bind the column matrix and initially passed directly through the column. The amount recovered in this nonbinding fraction represented 10% of receptors loaded onto the column, which indicates that 90% of the solubilized receptors were bound to the column matrix. The second range of recovered activity corresponded to receptors specifically eluted with a buffer containing 120 \(\mu\)M serotonin supplemented with lipid. This specific eluate was dialyzed to remove detergent and reconstitute the receptors into lipid vesicles. \(^{3}H\)-5-HT binding indicated that this specifically eluted and reconstituted fraction represented a 25% recovery of 5-HT-binding activity of the solubilized fraction initially loaded onto the column.

**Characterization of the Reconstituted Sample.** To characterize properties of the reconstituted proteins, \(^{3}H\)-5-HT binding assays were done over the range of 2–100 nM. The binding was saturable at 100 nM (Fig. 3A), and Scatchard analysis for a one-site model indicated an apparent \(K_d\) of 16.9 ± 3.7 nM (Fig. 3B). Specific binding of \(^{3}H\)-5-HT ranged from 80–90%. To further characterize the reconstituted binding site, competition assays were done using 5-methoxytryptamine (Fig. 4A), which labels 5-HT\(_1\) binding sites with high affinity (13). Bound \(^{3}H\)-5-HT at 5 nM was displaced by 5-methoxytryptamine over a concentration range of 2–50 nM with an apparent \(K_i\) of 27.4 ± 3.2 nM. The Hill slope of this competition was 0.77 (Fig. 4B). The 5-HT\(_1\)-specific ligand ketanserin (26) did not compete with 50 nM \(^{3}H\)-5-HT at concentrations to 4 \(\mu\)M (data not shown).

**Assessment of Enrichment in Affinity-Purified Reconstituted Sample.** The specific activity of reconstituted affinity-purified preparation in terms of maximum bound \(^{3}H\)-5-HT per mg of protein was determined and compared with that of crude membrane fragments. The limiting factor in the accuracy of this estimate was the protein concentration value. The reconstituted preparation was very low in protein concentration, and absorbance values for the reconstituted sample were consistently below the lowest point of the standard curve (0.05 mg/ml). However, by concentrating a pooled sample, a value of 0.012 mg/ml was determined for the reconstituted preparation. The maximum bound value was determined by extrapolation of Scatchard analysis data. For preparations where Scatchard analysis was not done, maximum bound was defined by the formula \(B_{\text{max}} = (L) + K_d/(L) \times B\), where \(B\) is the amount bound in pmol per ml.
centrated samples did not stain, presumably due to insufficient protein being loaded onto the gel. To achieve a concentration high enough for staining, the samples were concentrated by reverse dialysis against PEG followed by concentration using Centricon concentrators, although some questions arose concerning the efficacy of these concentrators, in that the reconstituted membranes seemed to be adsorbing to the concentrator filter thus resulting in a lower yield than expected. PEG reverse dialysis alone worked well in visualizing column fractions that had been specifically eluted with 3,4-dihydroxyphenylethylamine (dopamine; unpublished data). Specifically eluted serotonin fractions, concentrated ten to one hundred times, showed four diffuse bands with \( M_r \) ranges of 92,000–95,000; 78,000–84,000; 68,000–73,000; and 61,000–66,000 (Fig. 5).

**DISCUSSION**

An affinity column employing LSD covalently bound to an agarose matrix has been developed and used to purify serotonin receptors from bovine frontal cortex (Table 1). The purified receptors were reconstituted in artificial lipid vesicles made up of soybean lecithin (asolectin). The reconstituted receptors were similar, but not identical, to the pharmacological properties of 5-HT_1 type receptors identified in crude membrane fragments. The affinity-purified reconstituted samples exhibited a \( K_d \) of 17 nM, and the linearity of data indicated a one-site model as determined by Scatchard analysis. In crude membranes, a two-site model is often seen by Scatchard analysis (27) with \( K_d \) values that are between 1 and 5 nM and between 10 and 30 nM. The physical significance of this phenomenon is not known, and both apparent binding sites are considered 5-HT_1 sites. Because the 5-HT_1 receptor interacts with the GTP-binding protein, this two-site model seen in crude membranes could be due to subpopulations of the same receptor in different allosteric

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**Fig. 4.** Competition for [³H]5-HT binding (% bound) by 5-methoxytryptamine (5-MT). (A) Competition profile of 5-methoxytryptamine against H³-labeled 5-methoxytryptamine at 5 nM. (B) Hill plot of data is shown in A; the Hill slope is 0.77.

\( L \) is the concentration of [³H]5-HT (at 50 nM), and the \( K_d \) was 17 nM (Fig. 3B). Scatchard analysis of pooled fractions 70–72 showed a maximum bound value of 3.35 pmol per ml, which corresponds to an enrichment of 826-fold, but this fraction was not among the highest-activity fractions recovered. The highest-recovery fractions over two different column runs were 352 pmol per mg and 448 pmol per mg. These represent, respectively, enrichments of 1041 and 1324 compared with a maximum bound value of 0.338 pmol per mg (data not shown) in crude membranes. The purity of the receptor peptide in the reconstituted membranes is between 2.7 and 4.2% depending on the molecular weight of the ligand-binding peptide. This estimate does not include peptide subunits that may be necessary to maintain the structural integrity of the receptor, receptors oriented incorrectly in the reconstituted vesicles, or inactive receptors. Therefore, the actual purity is considerably higher.

**PAGE.** Active fractions were electrophoresed in the presence of NaDodSO₄ and stained with silver nitrate. Uncon-
conformations determined by the receptor’s interactions with regulatory elements. The one-site model seen in our affinity-purified sample could correspond to the “naked” receptor being reconstituted into the artificial lipid membrane without any regulatory components. NaDodSO₄ electrophoresis did not reveal any bands corresponding to the $M_r$ values of G protein subunits (42,000, 35,000, and 8,000), indicating no G protein subunits were coeluted with the reconstituted receptors. Also, in the NCB-20 neuroblastoma cell line, there is an adenylate cyclase-stimulating serotonin receptor that increases in affinity from $K_d$ of 150 nM to $K_d$ of 20 nM in the presence of gangliosides (17), which suggests a lipid component is involved in stabilizing the high-affinity state of the receptor. This lipid may not be present in asolectin, which could cause the slightly higher apparent $K_d$. The rank order of potency seen in the affinity-purified reconstituted sample of 5-HT > 5-methoxytryptamine >> ketanserin corresponds to the properties of 5-HT₁ receptors. This result suggests that 5-HT₁ receptors are the major component in our preparation but does not rule out the presence of other receptor types at lower concentrations.

NaDodSO₄ electrophoresis revealed four bands with $M_r$ ranges of 92,000–95,000, 78,000–84,000, 68,000–73,000, and 61,000–66,000. The identities of these bands cannot be directly assessed, but their possible identities as 5-HT receptors may be inferred from previous photolabeling experiments. A $M_r$ 63,000 photolabeled protein seen in the rat brain was identified as a 5-HT₁$_A$ receptor-binding subunit (28). Neuronal proteins with $M_r$ values of 45,000, 55,000, 63,000, 80,000, and 94,000 were labeled by a photosensitive azido compound that stimulates adenylate cyclase in Aplysia neurons, and the labeling was protected by the presence of serotonin during photolysis (29). Earlier photolabeling experiments also show the presence of a protein of $M_r$ 80,000 in rat brain that binds serotonin and LSD (30). With regard to the 5-HT₂ sites, an azidoazetanserin derivative was shown to label a protein of $M_r$ 69,000 in the rat brain (31). The approximate correspondences of these molecular weights with the bands we were able to detect by silver staining suggests that we have isolated proteins that are relevant to the serotonin receptor system. Our procedure can be scaled up to increase the yield for further purification.

In summary, we reconstituted serotonin-binding sites in highly purified form (~1000-fold compared with crude membrane) using an affinity column with covalently bound LSD molecule. The binding sites most likely correspond to a 5-HT₁ type receptor. NaDodSO₄ gel electrophoresis and Hill analysis of competition binding indicate the reconstituted receptor population may not be homogenous, and further pharmacological characterization is thus necessary.

We thank Prof. J. Konopelski for advice on the organic synthesis. We also thank Jenifer Dinsmore, Brian Strauss, and Anatol Sucher for technical assistance, and Dotty Hollinger for typing the manuscript.