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Solubilization and Characterization of the Serotonin 5-HT_{1c} Site from Pig Choroid Plexus

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SUMMARY

A novel type of serotonergic binding site, termed the 5-HT_{1c} site, was recently identified on choroid plexus epithelial cells. In the present study, we describe the solubilization of pig choroid plexus 5-HT_{1c} sites by the zwitterionic detergent 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS). High affinity labeling of both membrane-bound and solubilized 5-HT_{1c} sites was obtained by use of the serotonergic radioligand N1-methyl-2-[^{125}I]lysergic acid diethylamide (^{125}I-MIL). In solubilized preparations, ^{125}I-MIL exhibited a dissociation constant (K_d) of 2.0 nM and a 60–75% ratio of specific to total binding. Approximately 45% of the membrane binding sites were solubilized by CHAPS as measured by a postlabeling, polyethylene glycol precipitation method. The CHAPS-solubilized 5-HT_{1c} site fulfilled the accepted criteria for receptor solubilization. The affinities of a series of serotonergic antagonists for the 5-HT_{1c} site showed little or no change upon solubilization of the site. Serotonin, however, showed a 20-fold increase in affinity for the 5-HT_{1c} site after solubilization, which may indicate the loss of a modulatory component during detergent treatment. Both gel filtration and equilibrium sedimentation experiments indicate that the CHAPS-solubilized site is a large molecular weight complex. These studies demonstrate that the pig choroid plexus 5-HT_{1c} site can be solubilized with retention of its binding activity in a form suitable for further purification and characterization.

A new type of serotonin recognition site, termed the 5-HT_{1c} site, was recently characterized in rat and pig choroid plexus (1, 2). This site has been localized to the epithelial cell layer of this tissue (1). The rat choroid plexus 5-HT_{1c} site density is more than 10 times the density of any other serotonergic site in any tissue (1). Although the physiological role of the 5-HT_{1c} site has not yet been determined, it may be involved in the modulation of cerebrospinal fluid production or composition, which are primary functions of the choroid plexus.

The first steps toward molecular characterization of a neurotransmitter receptor are solubilization and purification of the receptor site. The serotonin 5-HT_2 receptor has been solubilized using the detergents CHAPS (3), digitonin (4), and lysolecithin (5). However, the solubilization yields were only 3–4% when assayed by a postlabeling methodology (3, 5). VandenBerg *et al.* (6) reported the solubilization of a high affinity [^{3}H]serotonin binding site, but this site did not exhibit stereoselective binding (7), and no further characterization or purification has been reported. The high density of 5-HT_{1c} sites and the relative simplicity of choroid plexus tissue make this a particularly attractive system for the solubilization and purification of a serotonergic recognition site.

We report here the solubilization and characterization of the pig choroid plexus 5-HT_{1c} site. Although the pig choroid plexus only exhibits 16% of the 5-HT_{1c} site density of rat choroid plexus (1), larger amounts of plexus tissue can be obtained from the pig. Solubilization of pig choroid plexus membranes with the zwitterionic detergent CHAPS resulted in the recovery of 45% of the serotonin 5-HT_{1c} sites. Pharmacological characterization of the solubilized 5-HT_{1c} site showed that it retains or increases its binding affinities for serotonergic compounds. A preliminary report of these findings has been published (8).

Materials and Methods

Homogenate preparation. Pig brains were obtained locally and placed on ice within 1 hr of slaughter. The choroid plexuses from 6–10 brains (approximately 3 g wet weight) were dissected from the lateral ventricles and homogenized in approximately 30 ml of ice-cold 0.32 M sucrose with 10–15 strokes in a Teflon pestle homogenizer rotating at approximately 500 rpm. The homogenate was centrifuged at 750 × *g* for 15 min at 4°. The supernatant was removed and centrifuged again as above, and the resulting supernatant was centrifuged for 20 min at 35,000 × *g* (17,000 rpm, Sorvall SS34 rotor). The pellet was resuspended

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ABBREVIATIONS: CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; ^{125}I-MIL, N1-methyl-2-[^{125}I]lysergic acid diethylamide; BSA, bovine serum albumin; PEG, polyethylene glycol; LSD, lysergic acid diethylamide.

in 15 ml of 50 mM Tris, pH 7.6, by homogenization and was incubated for 15 min at 37°. The homogenate was cooled and centrifuged at 35,000 × *g* for 20 min at 4°. The pellet was then resuspended by homogenization in 50 mM Tris, pH 7.6 (approximately 10 ml), to give a final protein concentration of approximately 1.5 mg/ml, and stored under liquid nitrogen. Protein was determined by the method of Lowry *et al.* (9), using BSA as the standard.

Solubilization procedure. Pig choroid plexus membrane homogenates were thawed and mixed with an equal volume of 20 mM CHAPS in 50 mM Tris, pH 7.6. The solution was vortexed occasionally during incubation at room temperature for 5 min (23°), then centrifuged at 110,000 × *g* (at r_{max}) for 1 hr at 4° in a Beckman Airfuge ultracentrifuge. ^{125}I-MIL binding activity in the solubilized supernatant was stable for at least 30 hr at 4°.

Binding assays. Solubilized extracts (25 μl) or membrane homogenates (diluted 1:1 with 50 mM Tris, pH 7.6, 25 μl) were incubated in triplicate with ^{125}I-MIL in 50 mM Tris, pH 7.6, 0.005% BSA (final volume of 150 μl) for 80 min at room temperature. After incubation, the solubilized binding sites were assayed by a modified PEG precipitation method (10). Samples were placed on ice and 20 μl of ice-cold 1% bovine γ-globulin in 50 mM Tris, pH 7.6, was added to each tube. After 5 min, 450 μl of ice-cold 23% PEG (Sigma PEG 8000) in 100 mM potassium acetate, pH 5.6, was added to each tube and the samples were vortexed. The samples were incubated on ice for 10 min, filtered under reduced pressure through Schleicher and Schuell glass fiber filters (No. 32, predipped in 7% PEG in 10 mM potassium acetate, pH 5.6), and washed rapidly four times with 5-ml aliquots of ice-cold 7% PEG in 10 mM potassium acetate, pH 5.6. Filters were dried for 30 min at 50° and counted by liquid scintillation spectroscopy. Specific binding was defined as that displaceable by 200 nM mianserin. Use of a gel filtration (Sephadex G50) method to separate bound from free radioligand gave results similar to those of the PEG precipitation method. ^{125}I-MIL concentrations were measured for each dilution used in the saturation binding experiments. For the competition studies (IC_{50} experiments), the ^{125}I-MIL concentration ranged from 0.5 to 2 nM. A buffer containing 50 mM Tris, pH 7.6, 0.005% BSA was used in all antagonist assays, and one containing 50 mM Tris, pH 7.6, 0.005% BSA, 10 μM pargyline, 5 mM ethylenediaminetetraacetate, and 1 mM sodium ascorbate, pH 7.6, was used for agonist assays. No differences were observed if the antagonist buffer was substituted for the agonist buffer in experiments with agonist compounds.

Gel filtration. The size of the solubilized ^{125}I-MIL binding site was estimated by gel filtration chromatography. Aliquots (150 μl) of the soluble extract were applied to an Ultrogel (LKB Instruments) AcA22 column (13.5 × 0.7 cm) equilibrated with 1.5 mM CHAPS in 50 mM Tris, pH 7.6. The sample was eluted with the same buffer at a flow rate of 3 ml/hr at 4°. Two drop fractions were collected and assayed in triplicate for specific ^{125}I-MIL binding by the PEG precipitation method. For prelabeling experiments, 150-μl aliquots of pig choroid plexus homogenate were incubated with ^{125}I-MIL in the presence or absence of 200 nM mianserin for 20 min at 37°. After incubation, the samples were placed on ice and then centrifuged at 25,000 × *g* for 5 min in the Airfuge ultracentrifuge. The pellet was washed two times with 200 μl of ice-cold 50 mM Tris, pH 7.6, resuspended by trituration for 1 min at room temperature in 150 μl of 10 mM CHAPS in 50 mM Tris, pH 7.6, and placed on ice. The sample was then centrifuged at 110,000 × *g* for 1 hr at 4°, and the soluble supernatant was removed and loaded onto the column. Two drop fractions were collected during the column elution and counted for radioactivity. Identical unlabeled samples were chromatographed and the fractions were assayed for protein by the amido black method (11), using BSA as the standard.

Airfuge equilibrium sedimentation. This procedure was adopted from the methods of Bothwell *et al.* (12) and Pollet *et al.* (13). Soluble choroid plexus extracts were diluted to a final concentration of 2 mM CHAPS in 50 mM Tris, pH 7.6, containing 5 mg/ml of dextran T40 (Pharmacia). Aliquots (150 μl) were centrifuged in a Beckman Airfuge ultracentrifuge at 16,500 rpm in 200-μl untapered polypropylene tubes for 30 hr at 4°C. Dextran T40 was included in all samples to generate a stabilizing density gradient during centrifugation. After centrifugation, the Airfuge rotor was slowly decelerated over 5 min and the top 50 μl of each tube was carefully removed with a capillary pipette, using a micromanipulator and dissecting microscope for accurate sampling. Control samples were stored in the dark for 30 hr at 4° without centrifugation. Triplicate samples from centrifuged and control preparations were assayed for total and nonspecific binding using the PEG precipitation method. A standard curve was generated by centrifuging proteins of known molecular weights for 30 hr at 16,500 rpm in a buffer containing 2 mM CHAPS and 5 mg/ml of dextran T40 in 50 mM Tris, pH 7.6, then assaying for the fraction of protein remaining (*F*) in the upper 50 μl using the Lowry method (9). The standard proteins chosen, and their respective (−log F) values were: thyroglobulin (0.585), apoferritin (0.418), and β-amylase (0.137). A centrifugation time of 30 hr has been shown to be sufficient to achieve sedimentation equilibrium under the conditions used in this experiment (13). Rotational rates (rpm) were determined by a digital tachometer, and the levitation air pressure was adjusted to obtain maximal rotor stability. Molecular weight values were determined by assuming a partial specific volume of 0.725 mg/ml for all proteins.

Ficoll gradient centrifugation. Aliquots of either the soluble extract or the membrane homogenate were layered onto a 4–13% linear Ficoll gradient containing 1.5 mM CHAPS in 50 mM Tris, pH 7.6, or 50 mM Tris, pH 7.6, alone. The Ficoll was deionized with AG501-X8D mixed bed resin (Bio-Rad) before gradient formation. Gradients were centrifuged for 18 hr at 40,000 rpm in a Beckman SW65 rotor at 4° and were fractionated manually from the top using a micromanipulator. Each fraction was assayed for total and nonspecific ^{125}I-MIL binding using the PEG precipitation method. For prelabeling experiments, aliquots of pig choroid plexus homogenate were incubated with ^{125}I-MIL for 20 min at 37° in the presence or absence of 200 nM mianserin. Samples were placed on ice for 5 min and centrifuged for 5 min at 25,000 × *g* at 4°. Pellets were washed two times with ice-cold 50 mM Tris, pH 7.6, and resuspended for 1 min at room temperature in either 10 mM CHAPS in 50 mM Tris, pH 7.6, or 50 mM Tris, pH 7.6, alone. Solubilized samples were centrifuged at 110,000 × *g* for 1 hr at 4°. Prelabeled solubilized extracts or homogenates were layered onto Ficoll gradients and were centrifuged and fractionated as above. Fractions were then assayed for radioactivity by liquid scintillation counting.

Materials. ^{125}I-MIL was synthesized and purified as described by Hoffman *et al.*[2] Bovine thyroglobulin, horse spleen apoferritin, sweet potato β-amylase, bovine γ-globulin, bovine pancreas trypsin, PEG 8000, CHAPS, and Ficoll 400 DL were obtained from Sigma Chemical Co. Proteinase K was obtained from Boehringer Mannheim Biochemicals. (+)- and (−)-LSD were obtained from the National Institute on Drug Abuse. All other drugs were obtained as gifts from the manufacturers.

Results

^{125}I-MIL, recently introduced as a high affinity serotonin 5-HT$_2$ radioligand (14), also exhibits high affinity[2] for the newly characterized choroid plexus 5-HT$_{1c}$ site (1, 2). Fig. 1 shows the saturation profile of ^{125}I-MIL binding to pig choroid plexus membrane homogenates. ^{125}I-MIL exhibited saturable, high affinity binding to the membrane preparation. The Scatchard plot of this binding data (Fig. 1, *inset*) was linear, indicating the presence of a single class of high affinity binding sites. The average dissociation constant (K_d) was 0.41 ± 0.02 nM and the site density (B_{max}) was 485 ± 37 fmol/mg of protein (n = 3). This site density agrees well with site densities of approximately 500 (1) and 300–600 fmol/mg of protein (2) reported for the 5-HT$_{1c}$ site in pig choroid plexus homogenates. Specific

[2] B. J. Hoffman, U. Scheffel, J. R. Lever, M. D. Karpa, and P. R. Hartig, submitted for publication.

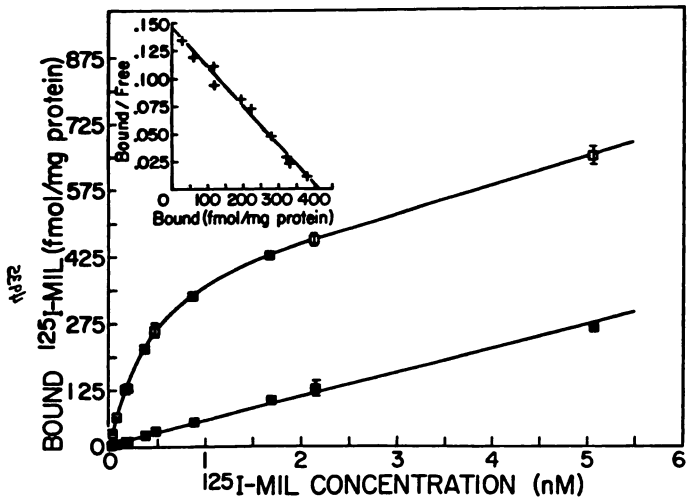

Fig. 1. Saturation binding of ^{125}I-MIL to pig choroid plexus membrane homogenates. Aliquots of pig choroid plexus membrane homogenate were assayed for specific ^{125}I-MIL binding as described in *Materials and Methods*. Total (□) and nonspecific (■) binding were determined by the presence or absence of 200 nM mianserin. The values shown are the combined results from two separate experiments and are expressed as means ± SD from triplicate assays at each concentration. The Scatchard plot of these data (*inset*) has a linear correlation coefficient, r^2, of 0.98 and a Hill slope of 0.98. The K_d and B_{max} values from this determination are 0.41 nM and 411 fmol/mg of protein.

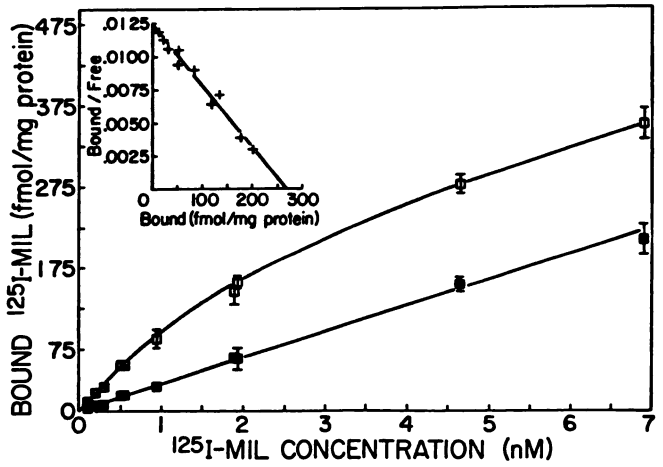

Fig. 2. Saturation binding of ^{125}I-MIL to the solubilized pig choroid plexus preparation. Aliquots of the CHAPS-solubilized extract were assayed for specific ^{125}I-MIL binding as described in *Materials and Methods*. Total (□) and nonspecific (■) binding were determined in the absence or presence of 200 nM mianserin. The values shown are the combined results from two separate experiments and are expressed as means ± SD from triplicate assays at each concentration. The Scatchard plot of these data (*inset*) has a linear correlation coefficient, r^2, of 0.97 and a Hill slope of 0.99. The K_d and B_{max} values from this determination are 2.17 nM and 268 fmol/mg of protein.

binding of ^{125}I-MIL to the membrane preparation was typically 75–85% of the total binding when a radioligand concentration of 0.4 nM was used in the assay.

Fig. 2 shows the saturation profile of ^{125}I-MIL binding to pig choroid plexus homogenates solubilized by the zwitterionic detergent CHAPS. Specific binding of ^{125}I-MIL to the CHAPS-solubilized sample was consistently 60–75% of the total binding, using a postlabeling PEG precipitation assay (see *Materials and Methods*). In comparison, prelabeling of the membrane sites with ^{125}I-MIL prior to solubilization at 4° produced greater than 90% specific labeling in the solubilized sample. Scatchard analysis of the postlabeling binding data (Fig. 2, *inset*) revealed a single class of high affinity binding sites. The average dissociation constant (K_d) was 2.0 ± 0.1 nM and the site density (B_{max}) was 293 ± 18 fmol/mg of protein ($n = 4$). Approximately 45% of the membrane binding sites and 70% of the membrane protein were solubilized by CHAPS. Increasing the ratio of detergent to protein did not increase the solubilization yield.

The competition of a variety of compounds for ^{125}I-MIL binding to the solubilized choroid plexus preparation is shown in Table 1. Only serotonin and serotonergic antagonists exhibited high affinity for the solubilized site. Mianserin was the most potent antagonist tested, showing an apparent inhibition constant (K_i) of 1.0 nM. The serotonergic antagonists ketanserin and cinanserin were also potent inhibitors of ^{125}I-MIL binding to the solubilized preparation. In contrast, dopaminergic and adrenergic antagonists were all very weak inhibitors of ^{125}I-MIL binding. In addition, the inactive (−)-isomer of LSD was at least 600 times less potent than (+)-LSD in competing for ^{125}I-MIL binding, demonstrating stereospecific binding to the solubilized site. The rank order of antagonist affinities at the solubilized pig choroid plexus site was identical to the rank order previously obtained for serotonin 5-HT$_{1c}$ sites in rat (1) or pig (2) choroid plexus homogenates (Spearman rank correlation coefficient $R_s = 1.0$, $n = 7$, $p < 0.001$). As shown in Table 1, all antagonist compounds tested in the solubilized pig choroid plexus preparation displayed very similar affinities in rat choroid plexus homogenates. In many cases, the displacement data for the solubilized and membrane sites was nearly superimposable, as shown in Fig. 3A for mianserin. The affinities of antagonist compounds for the solubilized pig choroid plexus sample were also in good agreement with values reported for inhibition of ^{3}H-mesulergine binding to pig choroid plexus homogenates (2). These data demonstrate that the CHAPS-solubilized ^{125}I-MIL binding site from pig choroid plexus is the same 5-HT$_{1c}$ site previously characterized using other radioligands in rat (1) and pig (2) choroid plexus homogenates.

TABLE 1

Competitive inhibition of binding in CHAPS-solubilized pig choroid plexus extracts and in rat choroid plexus membrane homogenates

CHAPS-solubilized extracts were assayed in triplicate for specific ^{125}I-MIL binding in the presence of various concentrations of the indicated agonists and antagonists. Bound ^{125}I-MIL was determined as described in *Materials and Methods*. Apparent K_i values were obtained from the Cheng-Prusoff relationship: $K_i = IC_{50}/(1 + L/K_d)$. Each value represents the mean ± SE for 3–6 determinations. Apparent K_i values for ^{125}I-LSD binding to rat choroid plexus homogenates (1) are provided for comparison.

Displacer	Apparent K_i values (nM) in:	
	Pig soluble extract	Rat membrane homogenate
Mianserin	1.0 ± 0.1	1.9 ± 0.2
(+)-LSD	7.5 ± 1.0	4.4 ± 0.4
Ketanserin	24 ± 2	28 ± 1
Cinanserin	71 ± 10	74 ± 2
Spiroperidol	448 ± 93	ND[a]
Propranolol	740 ± 63	736 ± 17
Phentolamine	1,280 ± 112	1,250 ± 100
Haloperidol	2,640 ± 321	2,050 ± 120
(−)-LSD	>5,000	>5,000
Serotonin	2.1 ± 0.4	30.4 ± 3.6
Dopamine	22,400 ± 8,750	43,100 ± 4,100
Epinephrine	129,300 ± 7,800	330,000 ± 12,300

[a] ND, not determined.

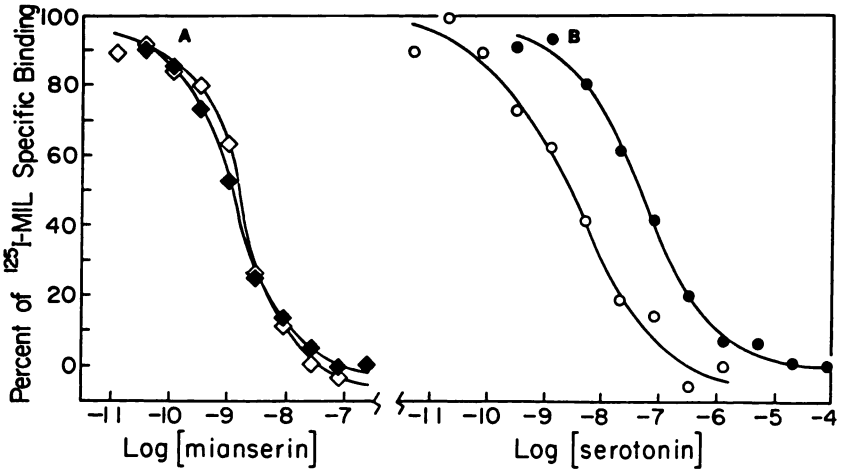

Fig. 3. Representative competition curves for inhibition of ^{125}I-MIL binding to solubilized and membrane sites. A. Inhibition of binding to solubilized (◇) and membrane (◆) sites by mianserin. B. Inhibition of binding to solubilized (○) and membrane (●) sites by serotonin. Data points represent averages of triplicate determinations from a single experiment. Nonspecific binding was approximately 11% of total binding in the membrane experiments and 40% of total binding in the experiments with solubilized samples. The pseudo-Hill slopes for inhibition of binding to the membrane and solubilized sites were 0.91 and 1.05 for mianserin and 0.73 and 0.62 for serotonin.

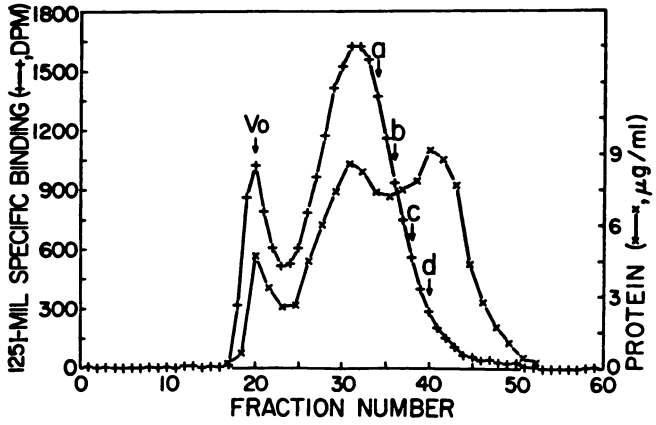

Fig. 4. Gel filtration chromatography of the CHAPS-solubilized pig choroid plexus preparation. Aliquots (150 μl) of the CHAPS-solubilized extract were preincubated with 1 nM ^{125}I-MIL (see *Materials and Methods*) and applied to an Ultrogel AcA22 column (13.5 × 0.7 cm) equilibrated with 1.5 mM CHAPS in 50 mM Tris, pH 7.6, and eluted with the same buffer at a flow rate of 3 ml/hr. Sixty fractions (two drops each) were collected and counted for radioactivity. Eighty-seven per cent of the specific binding sites applied to the column were recovered in the eluted fractions. Very similar results were obtained using a postlabeling methodology. Unlabeled extracts were chromatographed and assayed for protein using the amido black method (11). Protein standards (along with their known molecular weights and Stokes radii) were: (a) thyroglobulin (669,000; 8.5 nm); (b) apoferritin (443,000; 6.1 nm); (c) β-amylase (200,000; ~5.1 nm); and (d) BSA (66,000; 3.5 nm). A Stokes radius of 9.9 nm was determined for the peak at fraction 32.

Among the agonists tested, serotonin was by far the most potent in inhibiting ^{125}I-MIL binding to the soluble extract, exhibiting an apparent dissociation constant (K_i) of 2.1 nM. In parallel experiments using pig choroid plexus membrane homogenates, an apparent K_i of 40 nM was obtained for the inhibition of ^{125}I-MIL binding by serotonin [in agreement with previous studies on 5-HT$_{1c}$ sites (1, 2)]. Representative competition curves for serotonin inhibition of binding to the solubilized and membrane sites are shown in Fig. 3B. The slopes of the inhibition curves are parallel but the solubilized site shows a 20-fold increase in affinity (shift to the left). In contrast, other agonist and antagonist affinities are essentially unchanged after solubilization. A correlation plot of K_i values for inhibition of ^{125}I-MIL binding to solubilized pig 5-HT$_{1c}$ sites (Table 1) versus ^{125}I-LSD binding to rat choroid plexus homogenates (from Ref. 1) for all compounds except serotonin (n = 9) yields a linear correlation coefficient of 0.999, demonstrating the excellent agreement between these two data sets.

At 0°, the dissociation of ^{125}I-MIL from solubilized serotonin 5-HT$_{1c}$ sites was very slow (15% dissociation after 24 hr) and linear over time. This slow dissociation rate allowed us to apply prelabeled solubilized sites to gel filtration columns for determination of the size of the CHAPS-solubilized 5-HT$_{1c}$ site. Pig choroid plexus homogenates were prelabeled with ^{125}I-MIL, chilled on ice, solubilized with CHAPS, and applied to a column at 0–4°. Fig. 4 shows the elution profile of specific ^{125}I-MIL binding sites from the AcA22 column. The prelabeled ^{125}I-MIL binding sites eluted as a broad peak with a Stokes radius of 9.9 nm, in comparison to globular proteins of known Stokes radii (Fig. 4, *arrows*). The Stokes radius of the ^{125}I-MIL binding site corresponds to a molecular weight of approximately 880,000, using globular proteins as standards. Nonspecific binding was very low in the eluted fractions (less than 10% of specific binding at the peak fraction) and essentially constant throughout the elution volume of the column. Very similar results were obtained using a postlabeling methodology. Aggregation phenomena and the presence of tightly bound detergent and lipid in the solubilized complex may contribute to the large apparent molecular size. In addition to the 9.9-nm peak, a fraction of the binding sites also eluted in the void volume of the column, and the relative proportion of these sites increased with continued use of the same column. This may be due to aggregation of proteins on the chromatographic matrix, as has been shown to occur in other systems (15). The void volume fraction did not dissociate to the 9.9-nm form following reapplication to the column.

An Airfuge equilibrium sedimentation method (12, 13) was used to obtain an additional estimate of the molecular size of the solubilized ^{125}I-MIL binding site. In this technique, the logarithm of the fraction of protein remaining in the upper 50 μl of each Airfuge tube at equilibrium is linearly proportional to the molecular weight of the protein. Since this is an equilibrium sedimentation technique, the results are independent of the shape of the protein. In our experiment, 150-μl aliquots of the solubilized preparation were centrifuged to equilibrium (30 hr at 16,500 rpm), and the fraction of specific ^{125}I-MIL binding remaining in the upper 50 μl was determined. Standard proteins having well characterized molecular weights were centrifuged under identical conditions, and the fraction of protein remaining in the upper 50 μl (F) was assayed. The solubilized choroid

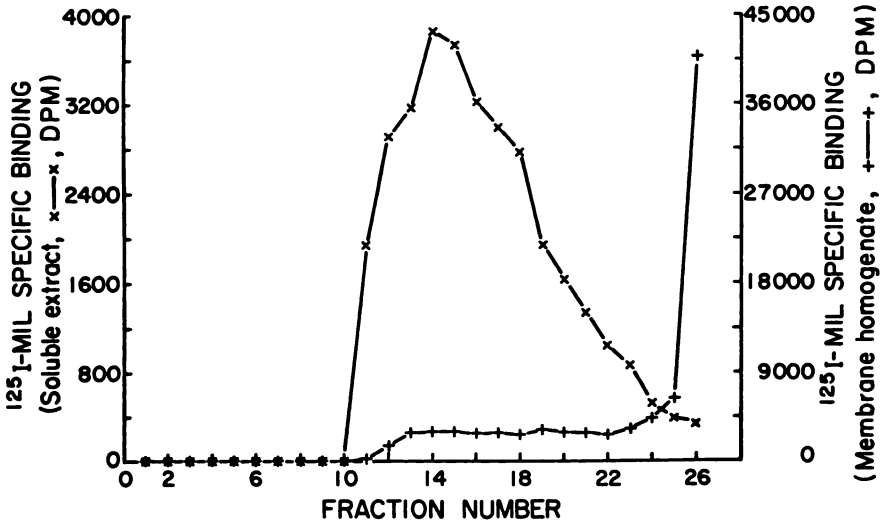

Fig. 5. Ficoll gradient centrifugation of the solubilized pig choroid plexus preparation. Pig choroid plexus membrane homogenates or solubilized extracts were layered onto 4–13% linear Ficoll gradients containing either 50 mM Tris, pH 7.6, or 1.5 mM CHAPS in 50 mM Tris, pH 7.6, respectively. Gradients were centrifuged for 18 hr at 117,000 × *g* in a Beckman SW65 rotor (4°), and fractions were collected from the top. Each fraction was assayed for total and nonspecific ^{125}I-MIL binding as described in *Materials and Methods*. Ninety-seven per cent of the specific binding sites applied to the gradient were recovered after centrifugation.

plexus 5-HT$_{1c}$ site yielded a (−log F) value of 0.742 which corresponds to a molecular weight estimate of 809,000. This value is in close agreement with that obtained from the gel filtration experiment.

The CHAPS-solubilized ^{125}I-MIL binding site meets the accepted criteria for receptor solubilization. This binding site did not sediment after centrifugation at 110,000 × *g* for 5 hr and was excluded from the void volume during gel filtration chromatography on an Ultrogel AcA22 column. The solubilized sites were not retained by small pore (0.22 μm) Millipore or Vanex cellulose acetate filters. The solubilized sites also were not retained by glass fiber filters unless PEG was added to induce precipitation. The solubilized extract and the membrane homogenate displayed different sedimentation characteristics on Ficoll gradients (Fig. 5). Whereas the solubilized sites remained near the middle of a 4–13% Ficoll gradient after centrifugation for 18 hr at 117,000 × *g* (at r_{ave}), the membrane homogenate sedimented to the bottom of the gradient. Nonspecific binding was low throughout the gradient, representing 12% of specific binding at the peak fraction in the solubilized sample gradient and 9% of specific binding at the peak fraction in the membrane sample gradient. The low level of specific ^{125}I-MIL binding sites found near the middle of the Ficoll gradient in the membrane sample appears to be associated with low density membrane vesicles. There are no "endogenous" solubilized sites in the membrane sample since an identical number of specific ^{125}I-MIL binding sites was collected in filtration assays performed in the presence or absence of PEG. In contrast, no specific binding could be detected in filtration assays of the solubilized sample unless PEG was added during the filtration step. Finally, specific ^{125}I-MIL binding to the solubilized extract was protease sensitive and exhibited increased thermolability in comparison to the membrane homogenate. Incubation of the solubilized extract with 10 μg/ml of trypsin or 10 μg/ml of proteinase K for 10 min at room temperature caused the specific ^{125}I-MIL binding to decrease by 60% and 40%, respectively. The increase in heat sensitivity of the solubilized extract was quite pronounced. Pretreatment of the solubilized extract for 3 min at 60° caused a loss of more than 95% of the specific ^{125}I-MIL binding compared to a loss of less than 5% in choroid plexus membrane preparations. These data clearly demonstrate that the high speed supernatant from CHAPS-treated pig choroid plexus membranes contains solubilized serotonin 5-HT$_{1c}$ sites.

Discussion

The pig choroid plexus 5-HT$_{1c}$ site can be efficiently solubilized by the zwitterionic detergent, CHAPS. The solubilized site meets accepted criteria for solubilization. It does not sediment following high speed centrifugation in low density media, it is excluded from the void volume during gel filtration chromatography, and it exhibits appropriate characteristics after Ficoll density gradient sedimentation. In addition, the solubilized ^{125}I-MIL binding site passes through 0.22-μm cellulose acetate (Millipore) filters, is not retained by glass fiber filters without PEG precipitation, is protease sensitive, and exhibits increased thermolability upon solubilization.

^{125}I-MIL, a newly characterized ergot radioligand (14), is a very effective label for the solubilized 5-HT$_{1c}$ site. ^{125}I-LSD can also be used to label 5-HT$_{1c}$ sites in pig choroid plexus homogenates (1) and in solubilized extracts, but ^{125}I-MIL exhibits both higher affinity and a higher specific to total binding ratio in solubilized and membrane preparations. ^{125}I-MIL exhibits saturable, high affinity binding to a single class of sites in both pig choroid plexus membrane homogenates and CHAPS-solubilized extracts. In the membrane preparation, the Scatchard plot of ^{125}I-MIL binding was linear over a 140-fold concentration range (0.036–5.08 nM) centered about the dissociation constant of 0.41 nM. Collection of accurate binding data over such a wide concentration range was made possible by the combination of a high 5-HT$_{1c}$ site density in the choroid plexus and the excellent ratio of specific to total binding displayed by ^{125}I-MIL. The linearity of this Scatchard plot indicates that only one serotonergic ^{125}I-MIL binding site (the 5-HT$_{1c}$ site) is present in this tissue.

Competition binding assays (IC_{50} experiments) were used to determine the affinities of a variety of agonists and antagonists for the CHAPS-solubilized 5-HT$_{1c}$ site. With the single exception of serotonin, the binding affinities of all compounds for the solubilized pig 5-HT$_{1c}$ site were in close agreement with values obtained in pig (2) and rat (1) choroid plexus membrane homogenates. In many cases, the values were the same within experimental error (Table 1). The slopes of the competition binding curves for both agonist and antagonist compounds were essentially unchanged after solubilization. Hill slopes were near 1 for the antagonists and approximately 0.7 for serotonin. Such close agreement between competition binding data at native and solubilized binding sites is not always seen in studies on

brain neurotransmitter receptors. In the case of dopamine D2 receptors, for example, the rank order of drug binding affinities is retained after solubilization, but the affinities are considerably lower at the solubilized site than in the native preparation (16, 17).

Serotonin was unique in exhibiting a 20-fold increase in affinity for the 5-HT$_{1c}$ site after solubilization. Selective alteration of agonist binding affinity was also observed when α-adrenergic (19, 20), β-adrenergic (20–22), and dopamine D2 receptors (17) were solubilized. Both increases (21, 22) and decreases (17–20) in agonist affinity were reported following solubilization, depending on the specific assay and solubilization conditions employed. In all cases, these agonist affinity changes arose from alterations in the interaction between the receptor protein and the GTP regulatory protein (the G protein). It is unlikely that a GTP regulatory protein is involved in the serotonin affinity change of the solubilized 5-HT$_{1c}$ site because the native 5-HT$_{1c}$ site does not appear to exhibit the GTP modulation of agonist affinity (1) characteristic of adenylate cyclase-linked receptor systems. It is possible, however, that another type of modulatory component is lost upon solubilization of the 5-HT$_{1c}$ site or that a selective conformational change occurs during solubilization. Selective conformational changes affecting agonist affinities have been observed in other systems. The acetylcholine receptor, for example, exhibits a selective agonist affinity change in association with the phenomenon of pharmacological desensitization (23). Solubilization of the acetylcholine receptor causes the receptor to undergo a conformational transition to the desensitized state (24). Whether a conformational change or the loss of a membrane component is responsible for the selective serotonin affinity change of the solubilized 5-HT$_{1c}$ site remains to be determined. Since this appears to be a selective affinity change for the natural agonist, serotonin, it is tempting to speculate that this affinity change may reflect a native process that occurs in the membrane.

The molecular size of the CHAPS-solubilized 5-HT$_{1c}$ site appears rather large in both equilibrium sedimentation and gel filtration studies (9.9 nm, approximately 850,000 daltons). Large molecular mass estimates ranging from 400,000 to 2,000,000 daltons have also been reported for other detergent-solubilized neurotransmitter receptors (15, 25). The large size of the solubilized 5-HT$_{1c}$ site suggests that protein association or aggregation may be occurring in the CHAPS extract. In addition, the binding of lipid and detergent to the complex may increase its apparent molecular size. It is clear, however, from the solubilization criteria discussed above, that the 5-HT$_{1c}$ site has been solubilized in a form suitable for further purification and characterization.

Approximately 45% of the pig choroid plexus 5-HT$_{1c}$ sites were solubilized by CHAPS as assayed by a postlabeling method. This compares with postlabeling yields of 3–4% for the solubilization of 5-HT$_2$ receptors from frontal cortex preparations (3, 5). The 5-HT$_{1c}$ site is the only serotonergic recognition site that has been efficiently solubilized with retention of its stereoselectivity, reversibility, and high affinity binding properties. Since this site is present at high densities in a relatively simple tissue, it should prove an excellent source for purification and characterization of a serotonergic site. Future studies on this solubilized site should serve to clarify both its molecular structure and its role in brain function.

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