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Isolation of a nicotine binding site from rat brain by affinity chromatography

(receptor/rat brain membranes/receptor purification)

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ABSTRACT **With the use of affinity chromatography, a [^{3}H]-nicotine binding site was purified almost 1,000-fold from a Triton X-100-solubilized extract of rat brain neural membranes. The affinity column was prepared by conjugation of (*R,S*)-6-(2-hydroxyethyl)nicotine to epoxy-activated Sepharose. Further purification of the material from the affinity column was resolved by using another column of the same affinity gel, resulting in the isolation of a major protein (about 95% purity) that had a M_r of 56,000, as determined by NaDodSO$_4$/polyacrylamide gel electrophoresis, with very minor components ranging in M_r from 47,000 to 83,000. With the use of various nicotine analogues, it was shown that the purified material exhibited nearly identical binding characteristics to rat brain membrane preparations, including stereoselectivity for the nicotine enantiomers. The K_d of the purified site, 3.5 $\times$ 10^{-9} M, was similar to that observed with membrane and Triton X-100-soluble preparations, whereas the binding capacity was >25 pmol/mg of protein, as compared to 0.07 pmol/mg of protein in the starting material. The results are discussed in relation to the purified nicotinic cholinergic receptor from electroplax. It was concluded that the nicotine site in rat brain was different from the cholinergic receptor of electroplax or calf skeletal muscle.**

A significant achievement in modern pharmacology and neurochemistry has been the isolation and characterization of the peripheral nicotinic cholinergic receptor (AcChoR) from the electric organ of *Torpedo* (fish) (1–3), electric eel (4, 5), and housefly brain (6). Various types of nicotinic cholinergic ligands, including α-bungarotoxin (BuTX) and bisquaternary amines, have been successfully employed in such studies, utilizing detergents such as Triton X-100 and desoxycholate to solubilize the receptor. The availability of the purified receptor has proven crucial to the understanding of receptor function and in characterizing the molecular nature of the reactive sites (7–12).

On the other hand, progress has been considerably slower in regard to the isolation and characterization of brain nicotinic receptors. In the past few years, numerous studies have focused on nicotine's neuropharmacologic and behavioral properties (13–23). Recently, there have been demonstrations of high-affinity nicotine binding sites in mammalian brain that exhibit a high degree of specificity for chemical structures related to the nicotine molecule and considerably lower affinity for nicotinic cholinergic antagonists and agonists (16, 24–28). Evidence has also been presented that the brain site's receptors may be noncholinergic (16, 25, 29).

Considerable difficulties are encountered in the isolation of brain nicotinic receptors. Although they appear to be widely distributed throughout the brain (24), the nicotinic receptors are located on neuronal membranes rather than on glands or effector organs as in the peripheral nervous system (10). Although BuTX has been successfully explored in peripheral AcChoR isolation, the specificity and properties of BuTX to nicotine neural receptors is to date undefined (7).

The present study reports on the use of affinity chromatography to purify to near-homogeneity a protein complex from rat brain membranes that appears to be the recognition site for nicotine. Previous isolations of nicotine peripheral receptors have utilized affinity ligands incorporating such substances as BuTX, decamethonium and related quaternary ammonium salts, gallamine, acetylcholine analogues, and *N*-methylpyridinium salts (9). Given the possibility that the brain nicotine sites are noncholinergic (16, 25, 29) and given the uncertainty regarding toxin binding specificities in nervous tissue, and affinity ligand was employed that incorporated the nicotine molecular structure (1). The integrity of the purified receptor preparation was established by comparing the binding properties of nicotine analogues (30–33) with the rat brain homogenate prior to and after the purification procedures.

MATERIALS AND METHODS

Solubilization of the Nicotine Receptor from Rat Brain. The procedure for solubilization of the nicotine receptor from rat brain neural membranes has been described in detail elsewhere (25). Briefly, it consists of homogenizing a suspension of a crude membrane preparation from rat brain in an ice-cold solution containing 50 mM Tris·HCl (pH 7.5) and 1% Triton X-100. Homogenization was performed in a tight-fitting glass homogenizer with a Teflon pestle for a period of about 10 sec, by using a drill press at a speed of 1,000 rpm. After incubation on ice for 15 min, the suspension was centrifuged at 100,000 $\times$ *g* for 30 min. The resulting supernatant was filtered and the filtrate was concentrated with an Amicon ultrafiltration cell (model 52) by using a PM-10 membrane.

Measurement of [^{3}H]Nicotine Binding. A number of procedures were used for the measurement of [^{3}H]nicotine binding. The procedure used for the Triton X-100-soluble extract was a modification of the method of Cuatrecasas (34). The incubation medium (in a 3-ml polycarbonate centrifuge tube) contained 0.1 μCi (1 Ci = 3.7 $\times$ 10^{10} Bq) of DL-[^{3}H]nicotine (60 Ci/mmol) and 10–100 μg of solubilized membrane protein in a final volume of 0.5 ml of 50 mM Tris·HCl (pH 7.5), with and without 0.01 mM nicotine. After incubation for 15 min in an ice bath, 0.5 ml of 1% bovine gamma globulin was added, followed by 0.5 ml of 25% polyethylene glycol (M_r 6,000). The contents were then vortexed and centrifuged at 5,000 $\times$ *g* in an Eppendorf centrifuge for 2 min. After aspirating the supernatant and carefully rinsing the side of the tube with 2 ml

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Abbreviations: AcChoR, peripheral nicotinic cholinergic receptor; BuTX, α-bungarotoxin.

of 50 mM Tris·HCl (pH 7.5), without disturbing the pellet, the bottom of the tube containing the pellet was cut off and radioactivity was determined by liquid scintillation spectrometry, using Aquasol (New England Nuclear) as the scintillation fluid.

[^{3}H]Nicotine binding to the purified material from affinity chromatography was also determined by the gel filtration procedure of Hummel and Dreyer (35). The purified material was applied to a Sephadex G-50 (course) column (0.5 × 15 cm) that was previously equilibrated and subsequently eluted with 50 mM Tris·HCl (pH 7.5) containing 0.5 nM [^{3}H]nicotine, with or without 1 μM L-nicotine. To minimize adhesion of the small amount of protein to glass, the column was pretreated with silicone; however, the results were unaffected by the treatment.

Preparation of Affinity Gel and Column. After 5 g of epoxy-activated Sepharose 6B was swollen in 100 ml of distilled H_2O for 1 hr, it was filtered by suction through a sintered glass filter and washed with 1 liter of H_2O. To a suspension of the gel in 2 ml of 0.02 M Na_2CO_3 (pH 10.5) was added 200 mg of (*R,S*)-6-(2-hydroxyethyl)nicotine (a gift of J. I. Seeman) and the contents were flushed with nitrogen gas and sealed. After incubation of the contents for 15 hr at 25°C with gentle shaking, Tris·HCl was added to a final concentration of 0.05 M and the incubation was continued for 1 hr. The contents were filtered and the gel was washed with 2 liters of H_2O to remove all traces of the ligand. The affinity gel was suspended in 100 ml of 50 mM Tris·HCl (pH 7.5) for 1 hr and poured into a 1.5 × 15 cm column that was then washed with 300 ml of 50 mM Tris·HCl. All operations were performed at 25°C.

After applying 5 ml of the solubilized protein solution containing 100 mg of protein in 50 mM Tris·HCl, pH 7.5/0.02% Triton X-100/0.1 mM phenylmethylsulfonyl fluoride/3 mM EDTA/pepstatin at 0.5 μg/ml, the column was eluted successively with 100 ml each of 50 mM Tris·HCl (pH 7.5) (fraction I), 0.1 mM nicotine in 50 mM Tris·HCl (pH 7.5) (fraction II), and again with 50 mM Tris·HCl and the protease inhibitors (fraction III); all solutions contained 0.1% Triton X-100. With the use of a fraction collector, the eluents were collected in 5-ml fractions at a rate of 0.4 ml/min and were combined into three separate fractions, I–III. Upon completion of the run, the column was washed with 100 ml of 50 mM Tris·HCl and was stored in the dark at 4°C until its reuse. It was possible to substitute 50 mM Na phosphate (pH 7.5) for Tris·HCl and eliminate the use of protease inhibitors without altering the final results.

After the fraction that was eluted with 0.1 mM nicotine was concentrated by flow dialysis and dialyzed against distilled water at 4°C, it was applied to a second affinity column identical to the first column. Elution was performed in a similar manner and the nicotine-eluted fraction again was concentrated by ultrafiltration, dialyzed against distilled H_2O, lyophilized, and stored in a desiccator at −20°C. The lyophilized material was then assayed for nicotine binding or used for NaDodSO$_4$/polyacrylamide slab gel electrophoresis, by employing the procedure of Laemmli and Favre (36).

RESULTS

Chromatographic Separation of Nicotine Binding Protein. Data on the various fractions obtained after applying the Triton X-100-solubilized membrane extract to the nicotine affinity column are presented in Table 1. The first fraction, eluted with Tris·HCl/Triton X-100, contained about 98% of the total applied protein, and the total binding was 70 × 10^{-14} mol of nicotine. Fraction II, which was eluted in the presence of 0.1 mM nicotine, contained a total of 10–30 μg of protein, with a total nicotine binding of 95 × 10^{-14} mol. Fraction III, eluted with 200 ml of Tris·HCl/Triton X-100, usually contained from 0.5–2 mg of protein, with only a trace of nicotine binding activity.

Table 1. [^{3}H]Nicotine binding and protein concentration of the fractions eluted from the affinity column

Fraction	Eluent	Volume, ml	Protein, mg	Total [^{3}H]nicotine binding, mol × 10^{-14}
I	Tris·HCl/Triton X-100	50	97–98	70
II	Tris·HCl/Triton X-100 and 0.1 mM nicotine	50	0.01–0.03 0.002–0.004*	95 6*
III	Tris·HCl/Triton X-100	200	0.5–2	1–2

A Triton X-100-soluble extract of rat brain membranes containing 100 mg of protein was applied to the affinity column. The fraction eluted with 0.1 mM nicotine was extensively dialyzed to remove the nicotine and binding was determined by the procedure of Hummel and Dreyer (35). Data are based on the first affinity column except for those designated *, which are derived from the second affinity column.

When NaDodSO$_4$/polyacrylamide gel electrophoresis was performed on the fraction eluted from the second affinity column with 0.1 mM nicotine, a major protein band with a M_r of 56,000 was observed along with minor components ranging in M_r from 47,000 to 83,000 (Fig. 1, lanes B and C). When this fraction was further fractionated on a second affinity column, the M_r 56,000 component was purified to near-homogeneity (Fig. 1).

K_d and Binding Capacity of Purified Receptor. The K_d for [^{3}H]nicotine binding remained constant after solubilization of rat brain membranes and purification by affinity chromatog-

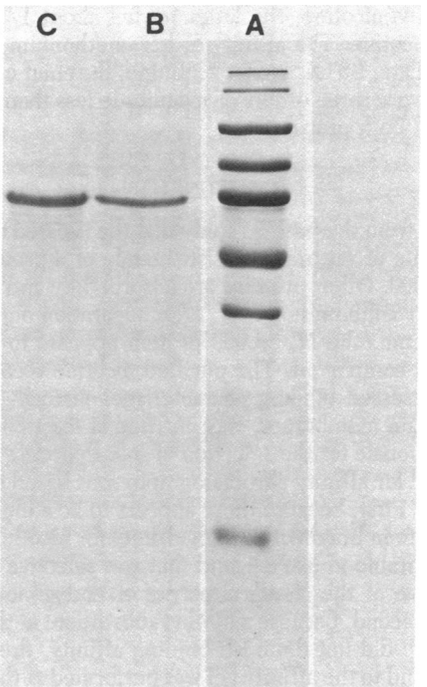

FIG. 1. NaDodSO$_4$/polyacrylamide slab gel electrophoresis of nicotine binding site. Lane A, M_r standards: phosphorylase a, M_r 94,600; bovine serum albumin, M_r 68,000; catalase, M_r 57,500; lactate dehydrogenase, M_r 35,000; trypsin inhibitor, M_r 21,500; lysozyme, M_r 14,400; and cytochrome *c*, M_r 12,000. Lanes B and C, two separate preparations eluted from second affinity column with 0.1 mM nicotine. Material was derived from 100 mg of protein.

Table 2. K_d and binding capacity of [^{3}H]nicotine to receptor preparations

Preparation	K_d	[^{3}H]Nicotine binding: Amount, mol/mg of protein	[^{3}H]Nicotine binding: Relative activity
Membranes	5.0×10^{-9}	4.0×10^{-14}	1
Triton X-100-solubilized membranes	6.0×10^{-9}	6.8×10^{-14}	1.7
Purified receptor	3.5×10^{-9}	2.4×10^{-11}	600–950*

The binding capacity for the purified receptor in five separate fractions ranged from 600 to 950 $\times 10^{14}$ mol/mg of protein. The K_d binding was obtained by Scatchard analyses as described elsewhere (25).
* Based on six separate experiments.

raphy (Table 2). The values were similar for intact and solubilized membranes as well as for the purified receptor, ranging from 3.5 to 6.0 $\times 10^{-9}$ mol/mg of protein. Upon solubilization of the membranes, a 70% increase in binding capacity was noted. The purified receptor represented a 600- to 950-fold enrichment of binding sites over the original membranes.

IC_{50} Values of Various Nicotine Analogues Against [^{3}H]Nicotine Binding. Various analogues were tested for their ability to compete for [^{3}H]nicotine binding to the purified receptor and were compared with the values for rat brain membranes (Table 3). Stereoselective binding was observed in the purified receptor as well as in the membranes, the affinity of the (*S*)-enantiomer of nicotine being almost 4 times greater than that of the (*R*)-isomer. As with the membrane preparation, the affinity of (*S*)-(−)-6-methylnicotine for the purified preparation was 3 times greater than that of (−)-nicotine. Comparable affinities to both preparations were noted for anabasine and 6-(2-hydroxyethyl)nicotine, the latter having about 1/20th the affinity of nicotine. The affinity of hexamethonium, 3-quinuclidinylbenzilate, BuTX, and (−)-cotinine, the chief metabolite of nicotine, were three orders of magnitude less than that of (+)-nicotine in both preparations.

Table 3. IC_{50} values of various nicotine analogues against [^{3}H]-nicotine binding to rat membranes and purified binding site

Drug	[^{3}H]Nicotine binding: Membranes: Amount, mol/mg of protein	[^{3}H]Nicotine binding: Membranes: Relative activity	[^{3}H]Nicotine binding: Purified receptor: Amount, mol/mg of protein	[^{3}H]Nicotine binding: Purified receptor: Relative activity
(*S*)-(−)-Nicotine	1.2×10^{-7}	1	1.0×10^{-7}	1
(*R*)-(+)-Nicotine	0.4×10^{-6}	3	3.5×10^{-7}	3.5
(*S*)-(−)-6-Methylnicotine	4.0×10^{-8}	0.33	3.0×10^{-8}	0.30
(*S*)-(−)-Cotinine	1.0×10^{-4}	830	7.1×10^{-5}	710
(*R,S*)-6-(2-Hydroxyethyl)nicotine	2.4×10^{-6}	20	2.5×10^{-6}	25
(*S*)-(−)-Anabasine	5.0×10^{-7}	5	2.0×10^{-7}	2
Hexamethonium	1.0×10^{-4}	830	3.0×10^{-4}	3,000
BuTX	2.0×10^{-4}	1,860	1.0×10^{-4}	1,000
3-Quinuclidinyl-benzilate	5.0×10^{-4}	4,150	2.0×10^{-4}	2,000

The concentration of [^{3}H]nicotine was 1 nM. [^{3}H]Nicotine binding to rat brain membranes was measured by a centrifuge assay, whereas binding to the purified receptor was measured by polyethylene glycol precipitation. The results are an average of three separate experiments agreeing within 8% of the mean.

DISCUSSION

It appears from the present study that the isolated nicotine recognition site of rat brain consists mainly of a protein having a M_r of 56,000. Other proteins are adsorbed by and eluted from the affinity column; however, after separation on a second affinity column, the M_r 56,000 protein appears to be the preponderant component. The purified nicotine recognition site, which possessed binding characteristics virtually identical to the rat brain membranes, was purified at least 800-fold.

The rationale for the selection of 6-(2-hydroxyethyl)nicotine as a ligand for affinity chromatography was based on a number of factors. First, because there appears to be a highly selective binding site in brain with a high affinity for nicotine analogues, it was desirable to use a ligand that was selective for this site, irrespective of the chemical nature of endogenous ligand for the site. Second, because a methyl substituent at the 6-position of nicotine did not diminish binding affinity, the conjugation of the ligand to the affinity gel was performed at the 6-position. The binding affinity of 6-(2-hydroxyethyl)nicotine for rat brain membranes and the Triton X-100-soluble extract was 1/20th that of (−)-nicotine, which had a K_d of 5×10^{-9} (25).

In previous studies based upon both the binding characteristics of rat brain membranes as well as pharmacologic studies, the [^{3}H]nicotine binding site was suggested to be noncholinergic (16, 25, 29). The AcChoR that has been purified from *Torpedo* appears to be a M_r 250,000-unit complex containing subunits of M_rs 40,000, 50,000, 57,000, and 66,000, with the M_r 40,000 subunit representing the acetylcholine site (37) and the M_r 66,000 unit presumably comprising the acetylcholine ionophore (38). It remains to be determined whether the M_r 40,000 or M_r 66,000 component of the *Torpedo* receptor has any similarities to the M_r 56,000 protein component from rat brain. Recently, it has been shown that a M_r 47,000 tryptic fragment of the M_r 66,000 subunit appears to contain a recognition site for the local anesthetic trimethisoquin (39). From a comparison of molecular weights, neither the individual proteins nor the [^{3}H]-nicotine binding complex purified from rat brain resembles the acetylcholine receptor of the electric organ. Before any definitive conclusions can be reached, comparative chemical, immunological, and binding studies need to be performed on both preparations.

Because tryptic or chymotryptic digestion of rat brain membranes destroys only 50% of the nicotine binding (16), the recognition site may reside in a fragment of the M_r 56,000 protein that could conceivably be related to the M_r 47,000 fragment or the M_r 40,000 subunit of the AcChoR from electroplax. In addition to the molecular weight differences between the brain nicotine site and the acetylcholine receptor, there are also differences in the binding characteristics of the two receptors. Whereas the electroplax receptor exhibits a high affinity for BuTX as well as for nicotinic cholinergic agonists and antagonists, the affinity of the brain nicotine receptor for such ligands is orders of magnitude less. It is of interest that [^{3}H]acetylcholine binding to rat brain membranes, BuTX and other antagonists, as well as nicotine cholinergic agonists were competitive only at excessive concentrations (K_i = 0.1 mM) (40). Recently, the AcChoR was purified from fetal calf muscle and found to contain four peptides bearing close resemblance to the material from electroplax. Therefore, the differences in the protein composition between the nicotine and acetylcholine receptors are not likely to be due to differences in species (41).

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