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The Binding of L-[^{3}H]Nicotine to a Single Class of High Affinity Sites in Rat Brain Membranes

PATRICK M. LIPPIELLO and KAY G. FERNANDES

Biochemical/Biobehavioral Group, Research and Development Department, R. J. Reynolds Tobacco Company, Winston-Salem, NC 27102

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SUMMARY

The binding of optically pure L-[^{3}H]nicotine to rat brain membrane preparations was studied using a rapid filtration method. The binding properties observed depended on the method used for tissue isolation. The most consistent results were obtained with membranes prepared in the presence of protease inhibitors, without divalent cations. Binding was saturable, reversible, and stereospecific. Scatchard analysis revealed a single class of high affinity sites with an average K_D of 2 nM and a B_{max} of approximately 200 fmol/mg of protein. The Hill coefficient was near unity. The K_D calculated from the kinetic rate constants for association (k_1 = 0.012 min^{-1} nM^{-1}) and dissociation (k_{-1} = 0.04 min^{-1}) was around 3 nM, in good agreement with the dissociation constant determined from equilibrium binding. In competition studies, cholinergic agonists were generally the most effective in inhibiting L-[^{3}H]nicotine binding, whereas antagonists were relatively ineffective. The D-isomer of nicotine was about 60-fold less potent than the L-isomer in inhibiting binding. The results were unaffected by temperature, with the exception that B_{max} was somewhat lower at 37°. The equilibrium binding properties of these sites were essentially identical in adult male and female brain. However, B_{max} was lower in fetal brain tissue. The present findings are consistent with the idea that there is a single class of high affinity nicotinic binding sites in rat brain with cholinoceptive properties.

The nicotinic acetylcholine receptor at the mammalian neuromuscular junction has been studied extensively, and much is now known about its structure and function (1–4). The characterization of this receptor was greatly facilitated by the availability of the pseudo-irreversible antagonist, α-bungarotoxin. Although it has been known for some time now that nicotine also binds to brain tissue (5), nicotine receptor sites in the brain have been difficult to characterize, partly due to the lack of a suitable ligand. There has been some evidence suggesting that α-bungarotoxin may also provide information on cholinergic systems in the brain. A high affinity α-bungarotoxin-binding site, having properties similar to those of nicotinic acetylcholine receptors in muscle, has been identified in and isolated from brain (6–8). However, the significance of this binding has been questioned since α-bungarotoxin fails to block neurotransmission in several neural systems (9–11), and classical nicotinic antagonists do not effectively inhibit its binding *in vitro* (12, 13). In addition, the regional distribution of α-bungarotoxin binding in the brain is different from that of either acetylcholine or nicotine (13, 14).

More recent studies have utilized radiolabeled nicotinic and cholinergic agonists to define the properties of nicotinic receptor sites in the brain, with varying results. For example, in rat brain [^{3}H]acetylcholine appears to bind to a single class of high affinity sites with nicotinic properties (15). However, studies using [^{3}H]nicotine have yielded conflicting results. There is evidence suggesting that this ligand binds to either one (13) or two (16) classes of receptor sites in mouse brain, and to one (17), two (18–20), or as many as five (21) classes of sites in rat brain. The apparent affinities of these sites for nicotine have ranged from 1 nM to 20 μM. Possible cooperative interactions among nicotinic receptor sites have been implicated as well (20, 21). Unfortunately, the significance of these results has been difficult to assess because of possible species differences, as well as differences in the types of assays and the assay conditions used. In addition, most studies on nicotinic binding sites in the brain have utilized a racemic mixture of DL-[^{3}H]nicotine. The interpretation of results based on the use of this nicotine may be further complicated by the apparent lack of stereospecificity at putative low affinity sites (13).

Recently, an optically pure preparation of the naturally occurring isomer of nicotine, L-[^{3}H]nicotine, has become available. Using this ligand, Abood *et al.* (20, 22) have described two classes of high affinity binding sites in rat brain membranes. These studies were based on a centrifugation assay. In the present studies, we have used L-[^{3}H]nicotine to characterize the properties of nicotinic receptor sites in rat brain, using a filtration assay. This assay, originally developed by Romano and Goldstein (18), is both rapid and precise and provides suitable controls for nonspecific binding. We also chose to

ABBREVIATIONS: EDTA, ethylenediaminetetraacetic acid; TMA, tetramethylammonium; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

include protease inhibitors in the preparative buffers to guard against possible binding artifacts created by proteolysis during the membrane isolation procedures. There is evidence, for example, that the binding properties of the acetylcholine receptor from *Torpedo* may be affected by proteolytic factors that are present during isolation procedures (23). We now have evidence that suggests this may also be true for nicotinic receptors in the brain. The most consistent results were obtained when protease inhibitors were utilized. Based on the present methodology, the results suggest that nicotine interacts with a single class of high affinity binding sites in rat brain.

Materials and Methods

Animals. Sprague-Dawley male (200–300 g), female (100–200 g), and timed pregnant female rats were purchased from the Harlan Company (Indianapolis, IN). Animals were housed separately in hanging stainless steel wire cages and were maintained on a 12-hr light/dark cycle (7 a.m.–7 p.m.). They received standard Purina Rat Chow (no. 5001) and water *ad libitum*.

Tissue preparation. Rats were anesthetized with 70% CO_2 prior to killing by decapitation. In experiments with adult animals, brains were removed immediately following decapitation. In experiments with timed pregnant females, the brains from 6–10 fetuses (20 days gestation) were rapidly removed and pooled following decapitation. Membranes were prepared from brain tissue according to the methods of Romano and Goldstein (18), with some modifications. Whole brains were removed, rinsed with ice-cold buffer, and homogenized at 0° in 10 volumes of buffer (w/v) using a Brinkmann Polytron, setting 6, for 10 sec. The preparative buffer consisted of Na_2HPO_4, 8 mM; KH_2PO_4, 1.5 mM; KCl, 3 mM; NaCl, 120 mM; EDTA, 2 mM; Hepes, 20 mM; iodoacetamide, 5 mM; and PMSF, 0.1 mM (pH 7.4 at 0°). The homogenate was sedimented by centrifugation (20 min; 50,000 × *g*; 0°). The pellet was resuspended in distilled, deionized water (5%, w/v) and incubated for 1 hr on ice. Membranes were recovered by centrifugation, as above, and resuspended in the assay buffer at a concentration of 1 mg/ml. The composition of the standard assay buffer was the same as the preparative buffer, with the addition of $MgCl_2$, 1 mM, and $CaCl_2$, 2 mM, and the elimination of EDTA, iodoacetamide, and PMSF.

Equilibrium binding assays. Routine assays were performed in polyethylene test tubes which had been presoaked in methanol to eliminate possible trace nicotine contamination. The assay mixture typically consisted of 200–400 μg of membrane protein in a final incubation volume of 500 μl. The concentration of nicotine was varied either from 0.1 to 200 nM using only L-[3H]nicotine, or 0.1 to 1000 nM (0.1–50 nM using L-[3H]nicotine and 50–1000 nM using 50 nM radiolabeled nicotine plus unlabeled L-nicotine). Incubations were carried out at 0° (2 hr) in a temperature-controlled cryobath (Exacal Ex-700), at room temperature (23°) for 20 min or at 37° (5 min) in a shaking water bath. At each temperature the incubation times were those found to be sufficient to achieve maximal binding. Assays were initiated by addition of the membrane suspension with rapid mixing. Blank incubations contained either 1 mM L-nicotine salicylate or 1 mM carbamylcholine. Incubations were terminated by adding 5 ml of assay buffer (0°), followed by rapid filtration under vacuum through a double thickness (ca. 0.9 mm) of Gelman type A/E glass fiber filters, using a Brandel multi-manifold tissue harvester. The additional thickness and smaller pore size of these filters (0.3 μm) facilitated maximal retention of particulate protein. Filters were always soaked overnight in 0.1% poly-L-lysine at 4°, to reduce nonspecific binding (18). Following the initial filtration of the assay mixture, filters were washed three times with ice-cold assay buffer (5 ml each), and air dried. Filters were then placed in counting vials and mixed vigorously with 20 ml of Scintiverse II (Fisher Scientific Co.), before quantification of radioactivity. Samples were counted in a Beckman LS-7000 liquid scintillation counter at 40–50% efficiency. All determinations were in triplicate. Specific binding was determined as the difference in binding between samples containing unlabeled L-nicotine salicylate (1 mM) or carbamylcholine (1 mM) and those which contained neither. Both compounds gave identical results.

Binding kinetics. The time course for nicotine binding was determined by incubating fixed concentrations of L-[3H]nicotine and membrane protein (250 μg) for varying amounts of time (0–1 hr), at 0°. Incubations were terminated at successive time points as described above. The time course for nicotine dissociation was followed by first incubating a fixed concentration of L-[3H]nicotine and membrane protein (250 μg) until equilibrium binding was achieved (1–2 hr) at 0°. This incubation was followed by the addition of an excess of unlabeled L-nicotine salicylate (1 mM), and rapid filtration and quantification of binding at successive times (0–1 hr) thereafter. For each time point in all of the kinetics studies, total binding and blanks (1 mM L-nicotine salicylate or carbamylcholine) were determined in triplicate. The kinetic constants for both association and dissociation were determined at several nicotine concentrations (2, 10, and 30 nM). The same results were obtained at all concentrations tested.

Competition studies. Membranes (200–400 μg of protein) were incubated with L-[3H]nicotine (15 nM) and at least eight concentrations of a given compound (0–10^{-2} M) in a total volume of 500 μl at 0° for 2 hr. Incubations were terminated and bound radioactivity was quantified as in equilibrium binding studies. In experiments with acetylcholine, 1.5 μM atropine sulfate and 100 μM physostigmine were included in the incubation mixture, to block muscarinic sites (15) and cholinesterase activity, respectively. Blanks were based on 1 mM L-nicotine salicylate. Values for 50% inhibition of L-[3H]nicotine binding (IC_{50}) were estimated graphically by linear regression analysis of log-logit plots. Inhibition constants were calculated using the equation, $IC_{50} = K_I\ (1 + [\text{nicotine}]/K_D)$.

Data analysis. Equilibrium binding data were analyzed using an iterative least squares curve-fitting algorithm called LIGAND (24). This program was adapted to an HP-86 microcomputer with minor modifications.

Analytical procedure. Protein was quantified by the method of Lowry *et al.* (25). The radiochemical purity of L-[3H]nicotine was routinely assessed by thin layer chromatography on silica gel G, using a solvent system consisting of $CHCl_3/C_2H_5OH/NH_4OH$ (82:25:0.25).

Materials. The following chemicals were obtained from Sigma Chemical Company (St. Louis, MO): L-lobeline hydrochloride, cytisine, L-nicotine, physostigmine, decamethonium bromide, hexamethonium bromide, carbamylcholine chloride, D-tubocurarine chloride, L-cotinine, iodoacetamide, PMSF, poly-L-lysine hydrobromide, polyethyleneimine, atropine sulfate, and α-bungarotoxin. DMPP and TMA were purchased from Aldrich Chemical Company (Milwaukee, WI). D-Nicotine was a kind gift from Dr. Hajime Kaneko (Japan Tobacco, Inc.). The salicylate salts of D-nicotine and L-nicotine were prepared by standard methods (26) and recrystalized from ethanol. Cotinine fumarate was prepared by Dr. T. Hudlicky, Virginia Polytechnic Institute (Blacksburg, VA). L-[3H]Nicotine (*N*-methyl-3H, specific activity 75.7 Ci/mmol), purity > 99%, was obtained from New England Nuclear Corporation (Newton, MA), stored frozen, and used within 1 month. Within 2–3 months, 5–7% of radiochemical impurities had normally accumulated, as judged by thin layer chromatography (13). Glass fiber filters, Gelman type A/E, were purchased from American Scientific Products (McGraw Park, IL).

Results

Equilibrium binding studies. When rat brain tissue was isolated in buffer which contained the protease inhibitors iodoacetamide and PMSF, without Ca^{2+} and Mg^{2+}, the specific binding of L-[3H]nicotine was found to be saturable (Fig. 1a). Binding was nearly maximal at a concentration of 50 nM. Between 0 and 40 nM, specific binding accounted for more than 90% of the total tissue binding. Binding to the glass fiber filters was about 5–10% of total binding when filters were presoaked

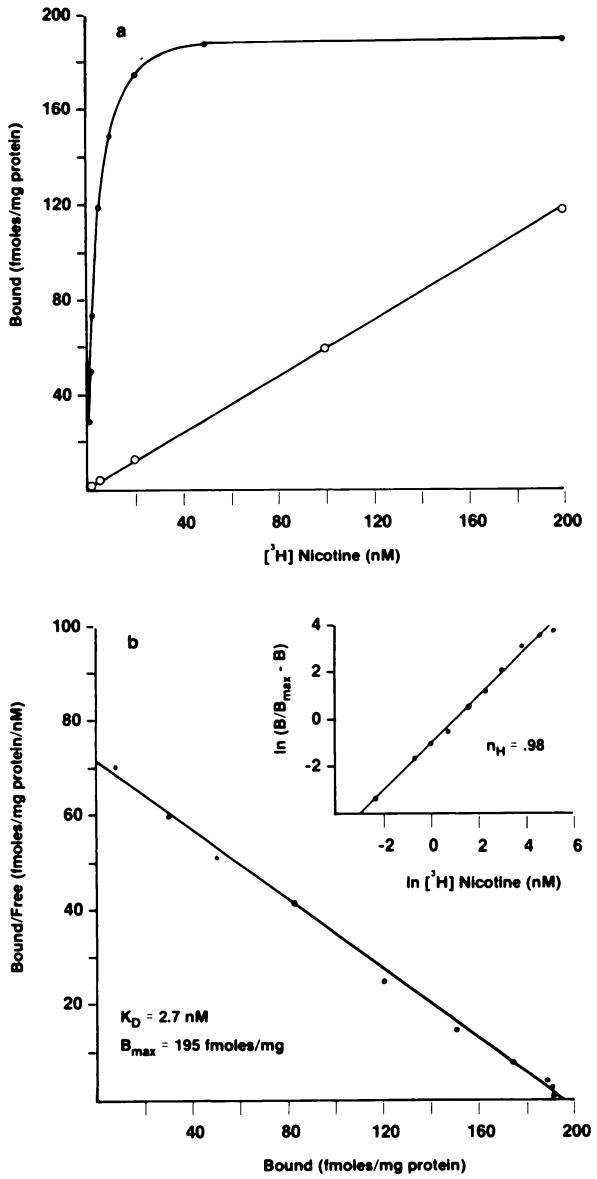

Fig. 1. Binding of L-[^{3}H]nicotine to rat brain membranes. a. Saturation binding of nicotine to membranes isolated from female brain tissue in buffer containing protease inhibitors (iodoacetamide; PMSF) and no divalent cations, as described in the text. Membranes (250 μg of protein) were incubated with L-[^{3}H]nicotine (0.1–200 nM) for 2 hr at 0°. Nonspecific binding was based on carbamylcholine blanks (1 mM). Specific binding (●) is the difference between total binding and nonspecific binding (○). b. Scatchard analysis of L-[^{3}H]nicotine binding. The data are representative of seven separate experiments. K_D and B_{max} were determined by linear regression. *Inset:* Hill analysis of the data. The Hill coefficient (n_H) was determined by linear regression.

in 0.1% poly-L-lysine, in agreement with previous observations (18).

Scatchard analysis revealed a single class of binding sites (Fig. 1b). In seven separate experiments the equilibrium dissociation constant (K_D) ranged from 1 to 3 nM, and the maximum binding capacity (B_{max}) varied between 180 and 220 fmol/mg of protein, with mean values of 2.0 ± 0.5 and 202 ± 15, respectively. The Hill coefficient (n_H) was always near unity (Fig. 1b, *inset*), indicating that there were no cooperative interactions among the sites. In experiments where the concentration range of nicotine was extended to 1000 nM, using unlabeled L-nicotine, the results were the same. There was no evidence of a second class of sites with lower affinity. The binding parameters were also unaffected by the choice of blanks. L-Nicotine salicylate (1 mM) and carbamylcholine (1 mM) both yielded identical results.

To determine the effects of temperature, the equilibrium binding properties of L-[^{3}H]nicotine were compared at 0°, room temperature (23°), and 37°, using brain membranes from adult females. There was no significant variation in the K_D between 0° and 37°, but the B_{max} was consistently lower when incubations were performed at 37° (Fig. 2). The K_D was also the same (ca. 2 nM) in membranes isolated from adult male brains or from fetal tissue at 20 days gestation (Fig. 2). However, the B_{max} for fetal tissue was about half that in adult tissue.

The results obtained depended on the conditions used to isolate the membranes. In experiments where membranes were isolated in buffer which contained no divalent cations or protease inhibitors, or in buffer which contained both protease inhibitors and divalent cations, the results were similar to those shown in Fig. 1 (Table 1). However, in experiments where brain tissue was isolated in the standard assay buffer (i.e., with Ca^{2+} and Mg^{2+}, without protease inhibitors), binding appeared to be saturable but did not reach complete saturation even at 400 nM (Fig. 3a). Nonspecific binding was typically 2–3 times higher, compared to membranes isolated with protease inhibitors. Scatchard plots were curvilinear, suggesting the possible presence of additional binding sites of lower affinity. The data were consistent with a two-site model, based on computerized curve-fitting analyses. A model based on three sites did not significantly improve the fit. The Hill coefficient was always significantly less than unity and ranged from 0.5 to 0.7. This may reflect, at least in part, negative cooperative interactions. However, since the B_{max} values were generally higher (300–400

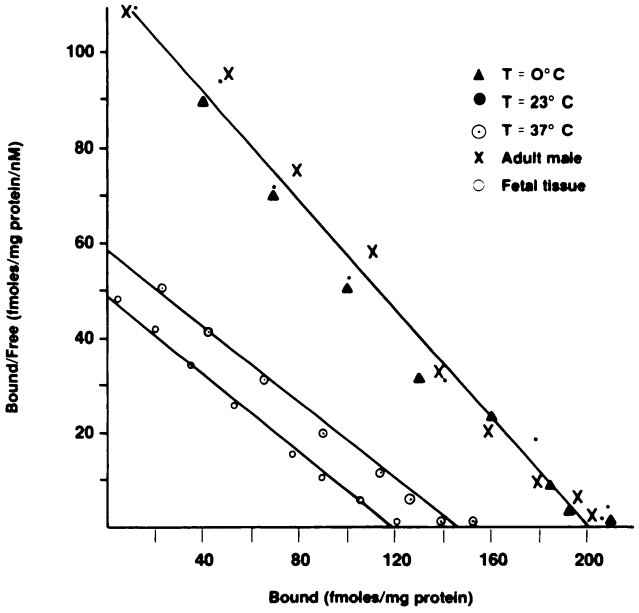

Fig. 2. Effects of temperature and tissue source on L-[^{3}H]nicotine binding. Membranes were isolated from male, female, and fetal brain tissue, using protease inhibitors and Ca^{2+}/Mg^{2+}-free buffer, as described in the text. Membrane protein (ca. 250 μg) was incubated with 0.1–200 nM L-[^{3}H]nicotine. For female tissue, incubations were at 0° (2 hr), 23° (20 min), or 37° (5 min). For adult males and fetal tissue, incubations were for 2 hr at 0°. The data presented are Scatchard plots representative of at least two experiments in triplicate in each case.

TABLE 1

Effects of membrane isolation procedures on the properties of L-[3H]nicotine binding

Rat brain membranes were isolated in standard assay buffer, with or without protease inhibitors (PMSF, iodoacetamide) or divalent cations, and assayed for [3H] nicotine binding, as described in the text. Binding parameters were determined using the LIGAND curve-fitting program (24). Values are the average of at least two experiments, done in triplicate, unless otherwise noted.

Preparative procedure	Number of sites	K_D	B_{max}	n_H[a]
		nM	fmol/mg	
Protease inhibitors + EDTA; no Ca^{2+}, Mg^{2+}	1	2	200	1
No protease inhibitors + EDTA; no Ca^{2+}, Mg^{2+}	1	1.9	192	1
Protease inhibitors plus Ca^{2+}, Mg^{2+} (N = 1)	1	2.6	190	0.9
No protease inhibitors plus Ca^{2+}, Mg^{2+}	2	1.8 100	190 80	0.6
Membranes[b] plus protease inhibitors (N = 1)	2	2.1 167	180 120	0.7

[a] n_H, Hill coefficient.

[b] Membranes were isolated in standard assay buffer containing Ca^{2+} and Mg^{2+}, and then incubated in the same buffer with the addition of PMSF and iodoacetamide, for 1 hr at 0°. Membranes were then recovered and resuspended in assay buffer for binding experiments.

versus 200 fmol/mg), the results are also consistent with the presence of additional binding sites. The apparent K_D for high affinity binding was always around 2–3 nM. The K_D for the low affinity binding component varied considerably between experiments and ranged from 50 to 200 nM.

In a control experiment, membranes were first isolated in standard assay buffer and then incubated with protease inhibitors before being assayed for [3H]nicotine binding (Table 1). The results were similar to those presented in Fig. 3, suggesting that the protease inhibitors were not themselves creating binding artifacts.

Binding kinetics. Studies on the kinetics of L-[3H]nicotine binding were conducted using membranes isolated with protease inhibitors. At 0°, the half-time for association was 3.5 min and equilibrium was reached within 60 min (Fig. 4a). The kinetics of association were biphasic and consisted of a rapid initial phase (0–5 min) followed by a slower phase thereafter (Fig. 4a, *inset). The* k_{obs} value, estimated from the initial slope of the log plot, was 0.4 min^{-1}. The association rate constant, calculated from the equation $k_1 = (k_{obs} - k_{-1})/[\text{nicotine}]$, was 0.012 min^{-1} nM^{-1}. The association rate constant (k_1) was also estimated from initial rates of binding (i.e., second order plots) in binding experiments using 2 nM L-[3H]nicotine, and was found to be 0.02 min^{-1} nM^{-1}.

The kinetics of nicotine dissociation were first order, with a half-time of 17.3 min (Fig. 4b). The dissociation rate constant (k_{-1}) was determined to be 0.04 min^{-1} (Fig. 4b, *inset*). The calculated equilibrium dissociation constant ($K_D = k_{-1}/k_1$) was 2–3 nM, in good agreement with the results of equilibrium binding studies. The kinetic binding parameters were not affected by the concentration of nicotine used. Experiments using 2 nM, 10 nM, and 30 nM gave identical results with the exception that 2–3 hr were required to reach equilibrium at the lowest concentration.

Inhibition binding. The pharmacological characteristics of the binding sites were studied, based on the ability of various compounds to inhibit the binding of L-[3H]nicotine. The results are presented in Fig. 5 and Table 2. All compounds tested, with the exception of α-bungarotoxin, blocked nicotine binding to

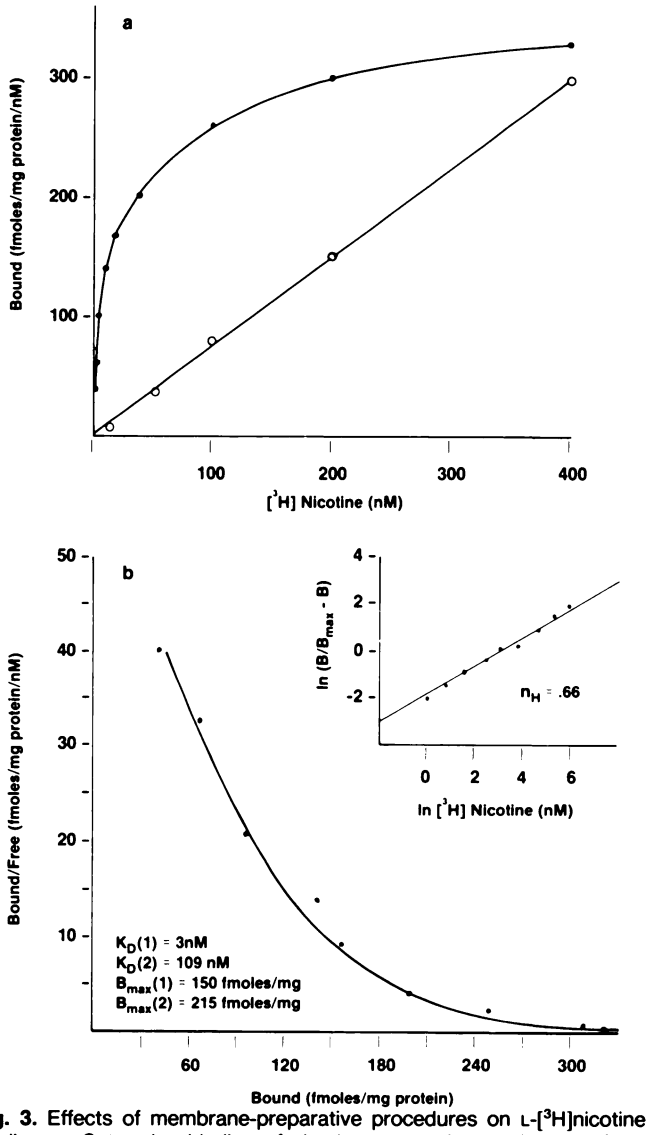

Fig. 3. Effects of membrane-preparative procedures on L-[3H]nicotine binding. a. Saturation binding of nicotine to membranes isolated from female brain tissue in standard assay buffer containing Ca^{2+} and Mg^{2+}, without protease inhibitors, as described in the text. Membranes (250 μg of protein) were incubated with L-[3H]nicotine (1–400 nM) for 2 hr at 0°. Nonspecific binding was based on carbamylcholine blanks (1 mM). Specific binding (●) is the difference between total binding and nonspecific binding (○). b. Scatchard analysis of L-[3H]nicotine binding. Apparent K_D values and B_{max} values were determined by nonlinear least squares curve-fitting analysis using a computerized method (LIGAND). *Inset:* Hill analysis of L-[3H]nicotine binding. The Hill coefficient (n_H) was estimated by linear regression.

the same extent. With respect to relative potencies, nicotinic agonists were more effective than antagonists in their ability to compete for nicotine binding. Cytisine, a potent ganglionic agonist, was 5–10 times more effective than L-nicotine. Acetylcholine was approximately equipotent with nicotine (K_I = 3 nM). Lobeline and DMPP were also effective inhibitors, as was carbamylcholine which had a K_I about 30-fold lower than that of L-nicotine. By comparison, tubocurarine, hexamethonium, and α-bungarotoxin, all potent antagonists peripherally, were at least 1000-fold less effective than most of the known agonists in competing for nicotine binding.

L-Nicotine salicylate was equipotent with the free base in competition studies. D-Nicotine was more than 60-fold less

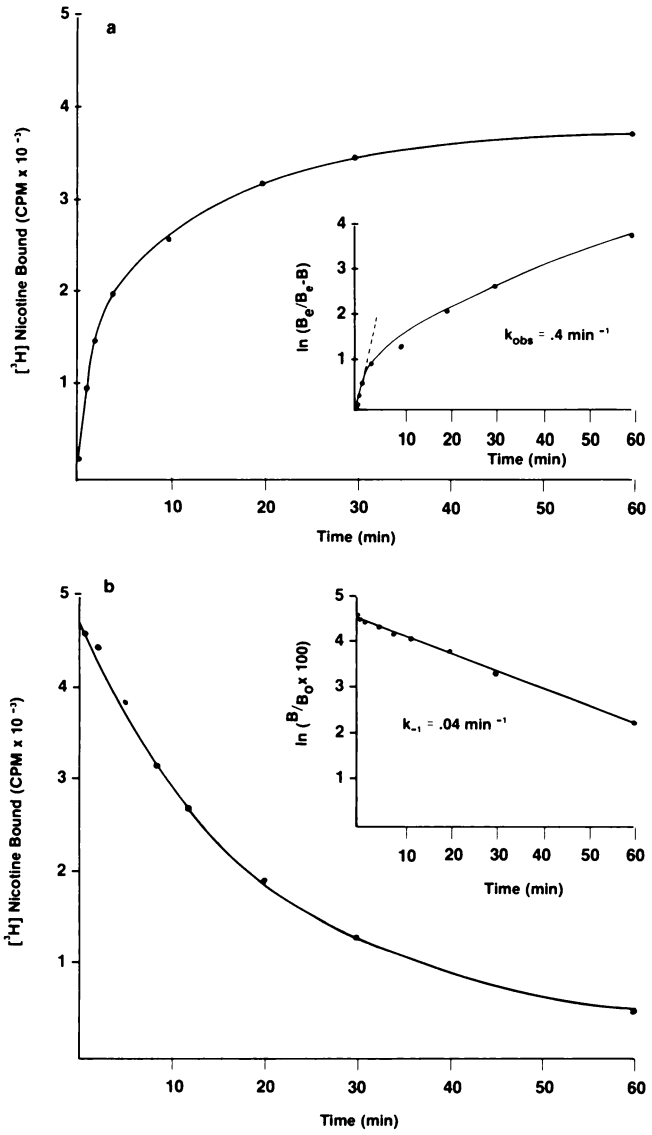

Fig. 4. Kinetics of L-[^{3}H]nicotine binding to rat brain membranes. Membranes were isolated from female rat brain tissue, using protease inhibitors, as described in the text. Incubations contained 250 μg of membrane protein and L-[^{3}H]nicotine (30 nM). Blanks were based on 1 mM L-nicotine salicylate. The results shown are representative of three experiments. a. The rate of association was determined from the specific binding at successive incubation times, measured after rapid filtration. Each time point, including carbamylcholine blanks (1 mM) was in triplicate. The k_{obs} was estimated graphically from the initial slope of the pseudo-first order kinetic plot (*inset*). b. The rate of dissociation was measured by first incubating for 2 hr at 0° and then adding 1 mM L-nicotine salicylate. Specific binding was determined at successive times thereafter, following rapid filtration. *Inset:* The off-rate (k_{-1}) was determined from the slope of the first order plot by linear regression.

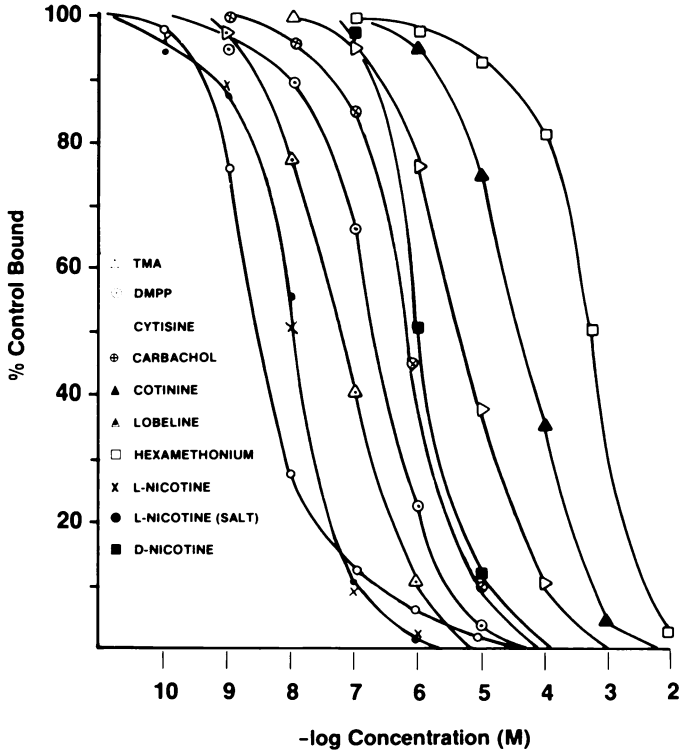

Fig. 5. Competition of various compounds for L-[^{3}H]nicotine binding. Membranes were isolated from female rat brain tissue using protease inhibitors, as described in the text. Membrane protein (250 μg) was incubated with L-[^{3}H]nicotine (15 nM) for 2 hr at 0°, in the presence of varying concentrations of a given compound (0–10^{-2} M). Specific binding is expressed as a percentage of control binding (i.e., no competing compound; 1 mM carbachol blanks). The curves shown are representative of at least two experiments with each compound, done in triplicate.

TABLE 2

Inhibition of L-[^{3}H]nicotine binding by various compounds

Membrane preparations from whole female rat brain (250 μg of protein) were incubated with L-[^{3}H]nicotine (15 nM) for 1 hr at 0° with varying concentrations (0–1 mM) of competing compounds, as described in the text. The concentration which inhibited [^{3}H]nicotine binding by 50% (IC_{50}) was estimated graphically, using a logit transformation. Inhibition constants (K_i) were calculated from IC_{50} values, using the method of Cheng and Prusoff (31); Hill coefficients were near unity for all compounds tested. The values shown represent the average of at least two separate experiments, each in triplicate. The variation between experiments was less than 20% for all compounds tested.

Compound	K_i
	nM
Cytisine	0.3
L-Nicotine (base)	2
L-Nicotine salicylate	3
Acetylcholine	3
Lobeline	9
DMPP	31
Carbamylcholine	100
D-Nicotine salicylate	125
TMA	375
Decamethonium	3,125
Cotinine fumarate	5,000
Tubocurarine	12,500
Hexamethonium	75,000
α-Bungarotoxin	>1 mM[a]

[a] Inhibition was 40% at 1 mM.

effective than the L-isomer, indicating that the binding sites possess a reasonable degree of stereospecificity. Cotinine, the principal metabolite of nicotine *in vivo*, was about 2500 times less effective than L-nicotine. The results of inhibition binding studies were the same, based on the calculated inhibition constants, regardless of the method used to isolate the membranes.

Discussion

There is still considerable disagreement in the literature with respect to both the number of classes of nicotinic receptor sites in the brain and their apparent affinity for nicotine. Based on the present results, it appears that nicotine may bind specifically and with high affinity to a single class of sites in the rat brain. This binding is saturable, reversible, and exhibits a

moderate degree of stereospecificity. Results from other laboratories also suggest that nicotine may bind to a single class of sites. For example, Schwartz *et al.* (15) reported a single class of sites with nicotinic properties in rat brain, using [^{3}H]acetylcholine as a ligand. Marks and Collins (13) have also presented evidence for a single class of high affinity sites in mouse brain, based on the binding of DL-[^{3}H]nicotine. Our data support these observations, with respect to the number of classes of sites. We have also noted several differences in the binding characteristics of L-[^{3}H]nicotine. For instance, the apparent K_D determined from the present experiments was always around 2–3 nM. This is considerably lower than that reported by Marks and Collins (13) for mouse brain (60 nM). By comparison, Schwartz *et al.* (15) reported a K_I of around 6 nM for the inhibition of [^{3}H]acetylcholine binding by L-nicotine in rat brain. We also found that the density of sites (B_{max}) was about twice that reported for nicotine-binding sites in mouse brain (13) or for acetylcholine sites in rat brain (15). However, this may simply be due to more efficient retention of membranes by type A/E filters.

The data also suggest that the binding properties do not change with temperature (Fig. 2), except for a decrease in B_{max} at 37°. The general lability of the sites at higher temperatures has been observed in studies with both rats and mice (13, 15). However, we did not see the appearance of additional low affinity binding components at 0° that was observed in mouse brain (13). The results also suggest that there is no difference between males and females in the binding parameters of these sites. However, there may be developmental changes in the density of sites, since the B_{max} for fetal tissue was about half that in adult brain (Fig. 2).

Several laboratories have suggested that there may be additional low affinity binding sites in the brain (18–22). However, the significance of such sites is unclear since the parameters determined for low affinity binding seem to vary widely, with K_D values ranging from 400 nM to 25 μM. We also noted similar variability in our results when membranes were isolated without protease inhibitors. By comparison, the properties of high affinity binding sites appear to be subject to less variability, even in studies where multiple sites have been observed. The equilibrium dissociation constants reported for rat brain have typically varied between 1 and 25 nM. Recently, Benwell and Balfour (27) reported a K_D of 3 nM for one of two classes of nicotinic sites they observed in rat brain. Similarly, Abood *et al.* (20, 22), using a centrifugation assay, found two classes of sites, one of which had a K_D of 2 nM for L-[^{3}H]nicotine binding. Regardless of the method used for membrane isolation, we consistently found a high affinity binding component with a K_D around 2 nM. In experiments where membranes had been isolated with protease inhibitors, it was the only binding component observed.

It is likely that many of the differences observed in nicotine binding sites, particularly those related to low affinity binding, can be accounted for by methodological variations. The present results suggest that the method used for isolating membranes from brain tissue may be a critical factor. For example, when membranes were isolated in buffer containing Ca^{2+} and Mg^{2+}, there was always evidence for additional low affinity binding sites. Occasionally Scatchard plots were convex upward between 0 and 1 nM nicotine, suggesting possible positive cooperativity effects as well. However, with the addition of protease inhibitors and the elimination of divalent cations, a single class of high affinity sites was always observed, and there was a significant reduction in nonspecific binding (cf. Figs. 1 and 3). Although protease inhibitors have been used in the isolation of nicotinic acetylcholine receptors from *Torpedo* (23) and from chick brain (28), they have not to our knowledge been utilized with mammalian central nicotinic receptors. Our data suggest that the appearance of low affinity sites may be due, at least in part, to the action of proteolytic factors which are present during membrane-preparative procedures. It is possible that a Ca^{2+}/Mg^{2+}-dependent neutral protease may be involved since the elimination of divalent cations from the preparative buffer gave similar results to those obtained with protease inhibitors (Table 1). This probably does not reflect irreversible ion-dependent aggregation or disaggregation of receptor subunits or other proteins during the isolation procedures since protease inhibitors were equally effective in the presence of both Ca^{2+} and Mg^{2+} (Table 1).

It is worth noting that the problems with interpretation of low affinity binding sites may also be compounded by the ligand. We found that the most consistent results were obtained when the [^{3}H]nicotine was used within 3–4 weeks. During this period, the purity was greater than 99%. However, after 2–3 months of storage, significant concentrations of radiochemical impurities (5–7%) were present, as judged by thin layer chromatography (13). Nonspecific binding to membranes was increased significantly, as reported previously by Romano and Goldstein (18). In addition, Scatchard plots became noticeably more curvilinear, resulting in increased K_D and B_{max} values (data not presented). To avoid similar problems from the oxidative deterioration of unlabeled L-nicotine, the more stable salicylate salt was used in all of the present experiments.

Using [^{3}H]acetylcholine, Schwartz *et al.* (15) found that the kinetics of association for nicotinic sites in rat brain could be described by a single, pseudo-first order process. By comparison, the kinetics of [^{3}H]nicotine binding appear to be more complex. In the present studies, the process of association was found to be biphasic at all concentrations of nicotine tested (2 nM, 10 nM, 30 nM). Sloan *et al.* (21) also found some evidence for this phenomenon in their studies with DL-[^{3}H]nicotine. It is possible that this reflects a rapid initial binding of nicotine followed by a slower conversion of the binding site to a high affinity, agonist-selective state. Slow conformational transitions between low and high affinity states have been suggested to explain the complex association kinetics observed with nicotinic acetylcholine receptors in *Torpedo* (29). Galper *et al.* (30) have postulated a similar mechanism to explain the biphasic kinetics of quinuclidinyl benzilate binding to muscarinic receptors in chick heart.

In the present studies the reversibility of L-[^{3}H]nicotine binding was confirmed by monitoring the rate of dissociation in the presence of excess unlabeled L-nicotine. We found that dissociation was described by a first order, single exponential process (Fig. 4b). We did not observe the biphasic dissociation kinetics reported by Sloan *et al.* (21) in their studies with DL-nicotine. If our data reflect the direct first order dissociation of nicotine from a high affinity complex, it would have to be concluded that an initial conformational transition of the binding site to this state, if it occurs, is essentially complete.

Based on the present competition studies, the overall pharmacological profile of the nicotinic sites suggests that they have

many cholinoceptive properties. Acetylcholine effectively inhibited [^{3}H]nicotine binding to the same extent as L-nicotine, and was equipotent in doing so ($K_I \simeq K_D$). Known nicotinic agonists, such as lobeline, TMA, and carbamylcholine were also found to be effective inhibitors of nicotine binding, in agreement with previous results (13, 15, 18). In addition, the amounts of specific binding and number of sites determined from L-nicotine blanks were identical with those determined from blank incubations with the cholinergic agonist, carbamylcholine. This was also observed by Marks and Collins (13) in mouse brain. If these sites are cholinergic, they also have properties which are unique and different from those of peripheral nicotinic cholinergic receptors. For example, classical nicotinic antagonists such as hexamethonium and tubocurarine were found to be relatively ineffective inhibitors. It has been suggested by Romano and Goldstein (18) that this reflects a shift in the receptor conformation to an agonist-specific state. We also found that α-bungarotoxin, a potent blocker peripherally, was unable to block L-[^{3}H]nicotine binding even at a concentration of 1 mM. However, there is now evidence from *in vitro* binding studies (13) as well as autoradiographic evidence (14) that nicotine and α-bungarotoxin bind to sites in the brain that have distinct and separate regional distributions. Clearly, the exact identity of central nicotinic receptors has yet to be completely defined and remains a goal for future studies.

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Send reprint requests to: Dr. P. M. Lippiello, Bowman Gray Technical Center, Building 611-13, Room 104W, R. J. Reynolds Tobacco Company, Winston-Salem, NC 27102.