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Nature of Nicotine Binding to Rat Brain P_2 Fraction[1]

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SLOAN, J W., G D. TODD AND W R MARTIN. *Nature of nicotine binding to rat brain P_2 fraction* PHARMACOL BIOCHEM BEHAV **20**(6) 899–909, 1984 — (−)−Nicotine may bind to as many as 5 sites in the rat brain P_2 preparation. A very high affinity site (K_D~2 2×10^{-11} M), a positive cooperativity site; a high affinity site (K_D~5 2×10^{-9} M); a low affinity site (K_D~4.5×10^{-5} M) and a very low affinity site The curvilinear nature of both Scatchard plots and kinetic curves indicates the presence of multiple binding sites Evidence for a positive cooperativity site includes (1) The configuration of Scatchard plots (at low concentrations) of saturation as well as inhibition curves for (−)− and (+)−nicotine. (2) The Hill number of 1.37 for the binding of low concentrations of (±)−[^{3}H]nicotine. (3) Selectivity among cholinergic drugs for producing positive cooperativity (4) Markedly different specificities of drugs for the positive cooperativity site. Thus while only (+)− and (−)−nicotine interacted with the very high affinity site, acetylcholine, atropine, mecamylamine, lobeline, carbachol, (+)−nicotine and (−)−nicotine enhanced the binding of (±)−[^{3}H]nicotine and cytisine, anabasine, cotinine and choline selectively inhibited binding at the high affinity site Several lines of evidence indicate that there is stereospecificity (+)−Nicotine was more potent than (−)−nicotine in inducing positive cooperativity whereas (−)−nicotine was 80 times more potent than (+)−nicotine in inhibiting binding at the high affinity site Further, the specificity of the binding sites can be altered by changing the concentration of the buffer which gives additional evidence for the lability of the nicotine binding site Although the pharmacologic significance of the different binding sites has not been determined, these data taken together indicate that (±)−[^{3}H]nicotine binds with specificity to multiple sites in the rat brain P_2 preparation with a complexity not addressed heretofore

Nicotine Multiple nicotine binding sites Lability of nicotine binding site High affinity binding site Positive cooperative binding site Nicotine stereospecificity Drug specificity

RADIO α-bungarotoxin has been used extensively to characterize nicotinic acetylcholine receptors in the rat brain [11, 15, 17, 21, 25–27, 32, 36, 47–49, 51, 53, 58] and with few exceptions [27,32] a single site has been observed. Although the regional distribution of [^{125}I]-α-bungarotoxin and [^{3}H]nicotine binding in the brain is similar [14, 49, 53, 60] this distribution differs from the localization of [^{3}H]acetylcholine binding [52] and shows major differences in binding characteristics. Both nicotine and acetylcholine are less potent in displacing [^{125}I]-α-bungarotoxin [11, 17, 26, 27, 36, 51, 53] than in displacing [^{3}H]nicotine or [^{3}H]acetylcholine from brain binding sites. Further, α-bungarotoxin has a low affinity for both [^{3}H]nicotine [1, 3, 4, 14, 43] and [^{3}H]acetylcholine binding sites in the rat brain [52]. Finally, there is a poor correlation between binding data and the ability of α-bungarotoxin to antagonize cholinergic function [2–4, 12, 13, 18, 24, 34, 35, 37, 38, 40–42]. Although nicotine and acetylcholine interact with an α-bungarotoxin binding site in the brain, they may also interact with other receptor populations.

Recent findings in the rat brain using nicotine as a radioligand are inconsistent. Two binding sites were found by some investigators using either whole brain crude mitochondrial or synaptosomal preparations [1, 14, 43, 56, 59, 60] while others have found one site [4, 31, 57]. Inconsistent findings have also been reported with the mouse brain. One site was found with a synaptosomal fraction using equilibrium dialysis at 4°C [50] whereas others have found two sites with both the crude membrane and P_2 preparation incubated at 21°C, using the filtration procedure [54]. Incubation conditions have been reported to alter the number of binding sites observed in the whole brain particulate fraction of the mouse brain. One site was found when incubation was carried out at either 20°C or 37°C whereas two sites were found at 4°C [30].

During the course of the present study it was found that the binding of (±)−[^{3}H]nicotine to the rat brain P_2 fraction was much more complicated than has been previously reported.

METHOD

Drugs and Chemicals

(±)−[^{3}H]nicotine (35.1, 63.1 or 71.2 Ci/mMole) was obtained from New England Nuclear (Boston, MA) and the purity checked periodically (>95%) with three TLC solvent

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systems (methanol.ammonium hydroxide, 99.1, silica gel, chloroform.methanol:diethylamine, 80.15:1, silica gel and methanol:acetic acid, 99·1, alumina). It was diluted to 2 μM with mercaptoacetic acid and stored at 4°C [43]. Other drugs and chemicals and their sources were. (−)−nicotine, (−)−cytisine and (−)−anabasine, Research Plus (Bayone, NJ); carbachol, atropine, (−)−lobeline, poly-1-lysine (type V), N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid (Hepes), Tris-HCl and DFP, Sigma Chemical Co. (St Louis, MO); acetylcholine chloride and choline chloride, Calbiochem-Behring (San Diego, CA). (+)−Nicotine was resolved by Dr. W T Smith and Miss Amy Howell (Chemistry Department, University of Kentucky). Other drugs were generous gifts from the following sources· Mecamylamine hydrochloride, Dr. Clement Stone, Merck Sharp and Dohme (West Point, PA); (−)−cotinine hydrochloride, Dr. Jeffery Seeman, Philip Morris Research Lab (Richmond, VA), one batch of (+)−nicotine, Dr E L. May, Medical College of Virginia (Richmond, VA) and one batch of cytisine, Dr. Leo Abood, University of Rochester Medical Center (Rochester, NY)

Membrane Preparation

Female Sprague-Dawley rats weighing 200–300 g were decapitated between 0800 and 0900 hr and a P_2 fraction was prepared from whole brain homogenates at 4°C The brains were homogenized in 10 volumes of 0.32 M sucrose using a glass vessel and ten strokes of a serrated teflon pestle (1600 rpm) and then centrifuged at 2000×g for 10 min The supernatant was transferred to weighed tubes and centrifuged for 20 minutes at 50,000×g. The resulting pellet was homogenized for 30 seconds in 50 volumes of 50 mM Tris-HCl buffer using a Brinkmann Polytron and recentrifuged at 50,000×g for 10 min. This final pellet was weighed and diluted in ice cold Hepes buffer (Hepes, 50 mM; NaCl, 118 mM, KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM and NaOH to pH 7.4) to a concentration of 80 mg of isolated P_2 membranes/ml of Hepes It was again homogenized with the Brinkmann Polytron. The tissue suspension was stirred magnetically in an ice bath. Aliquots (0.5 ml) were transferred to 12×75 mm polypropylene RIA tubes and held in an ice bath. Membrane aliquots were incubated with drugs (prepared in Hepes) in a total volume of 1 ml with a final tissue concentration of 40 mg/ml The experiments with acetylcholine differed in that the assay was conducted in the presence of DFP (1×10^{-4} M).

Drug Preparation

Drug solutions used in the inhibition studies were prepared the day of use in Hepes buffer The pH of the drug solutions were adjusted to 7.4 with NaOH and HCl. Serial dilutions were then made with Hepes buffer to obtain the appropriate concentration. All solutions were kept at 4°C and protected from light.

Binding Assay

All samples were assayed in triplicate and each inhibition study was usually repeated 4 times using different homogenate preparations. The tubes were stoppered and incubated for 60 min in a shaking ice bath (120 oscillations/min). A staggered starting time of 10 min was used for each successive triplicate set and all samples were vortexed for 10 sec at each 10 min interval For the (±)−[^{3}H]nicotine saturation studies each sample contained 0.5 ml of tissue suspension, 0.25 ml of the different concentrations of (±)−[^{3}H]nicotine solution and 0.25 ml of either Hepes (total binding) or 1×10^{-2} M (−)−nicotine. For the inhibition studies, samples contained 0 5 ml of tissue suspension, 0 25 ml of (±)−[^{3}H]nicotine (final concentration 2.4×10^{-8} M), and 0 25 ml of either Hepes, or the inhibiting drug to give the appropriate final concentrations. Non-saturable binding was determined in the presence of 1×10^{-2} M (−)−nicotine or a concentration of the particular drug under study that inhibited (±)−[^{3}H]nicotine binding to a degree comparable with 1×10^{-2} M (−)−nicotine [this concentration of (−)−nicotine was chosen since lower concentrations produced less inhibition and higher concentrations produced no further inhibition of binding]. At the end of the incubation period, 3.5 ml of ice cold Hepes was added and the suspension was filtered immediately at a reduced pressure (460–510 mm/Hg) using a Hoeffer filter apparatus (Hoeffer Scientific Instruments, San Francisco, CA) through Whatman GF/C glass fiber filters soaked overnight at 4°C in polylysine, 0.1%. Binding to filters was less than 10%. The incubation tubes were rapidly rinsed four times with 3.5 ml of cold Hepes and the rinse was used to wash the filter. The first filtration time was less than 5 sec and each wash was about 3 sec [10] The filters were dried by approximately 20 sec of suction and placed tissue-side up into counting vials. After the samples were transferred to the vials, 0.1 ml of 0.005 M HCl in absolute ethanol was added directly to the filters followed by 10 ml of scintillation fluor (198 g of napthalene, 19 3 g PPO; 0 175 g dimethyl POPOP dissolved in a mixture of 2250 ml p-dioxane and 450 ml of xylene) All reagents were scintillation grade The vials were vortexed thoroughly and counted three times in a Packard tricarb 300 C scintillation counter (Packard Instruments Co , Downers Grove, IL) interfaced with an Apple II Plus Microcomputer (Apple Computer, Inc , Cupertino, CA) A counting efficiency of 60% was obtained.

TABLE 1

ANALYSIS OF VARIANCE OF (−)-NICOTINE INHIBITION OF (±)-[^{3}H]NICOTINE BINDING*

ANOVA I Source of Variation	Degrees of freedom	Mean square	F ratio	$p <$
Homogenates	3	68×10^{-7}	1 93	$1\ 4\times10^{-1}$
Concentrations	14	3667×10^{-7}	104 74	6×10^{-28}
Regression	1	44877×10^{-7}	1282 21	7×10^{-12}
Deviations	13	495×10^{-7}	14 17	3×10^{-11}
Error	42	35×10^{-7}		
Total	59			

ANOVA II Number of Lines	Degrees of freedom	Mean square	F ratio	$p <$
1	58	207×10^{-7}	—	—
2	56	121×10^{-7}	1 71	$2\ 3\times10^{-2}$
3	54	116×10^{-7}	1 78	$1\ 7\times10^{-2}$

*The data are shown in Fig 6 and analyzed by ANOVA I and II as described in the Method section

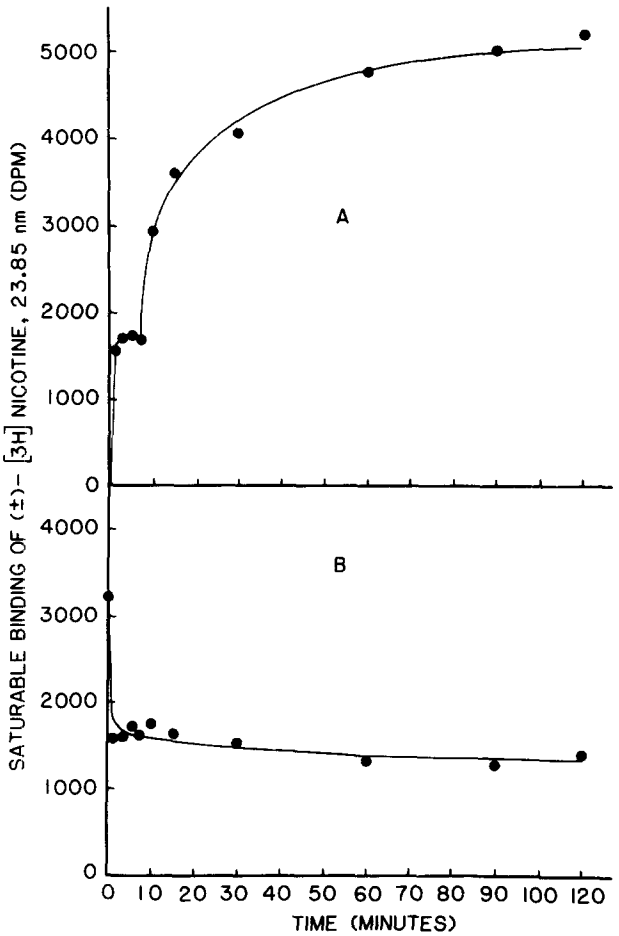

FIG 1 Time course of (±)−[³H]nicotine (23 85 nM) association with and dissociation from the rat brain P_2 fraction at 4°C. Each point is corrected for non-saturable binding Panel A Association curve obtained by filtering and washing the samples at the times indicated Panel B Dissociation curve obtained by pre-incubating the P_2 preparation with (±)−[³H]nicotine for 60 min prior to adding 10 mM (−)−nicotine The samples were further incubated until the reaction was stopped by filtration at the times shown. Each point is the mean of triplicate determinations

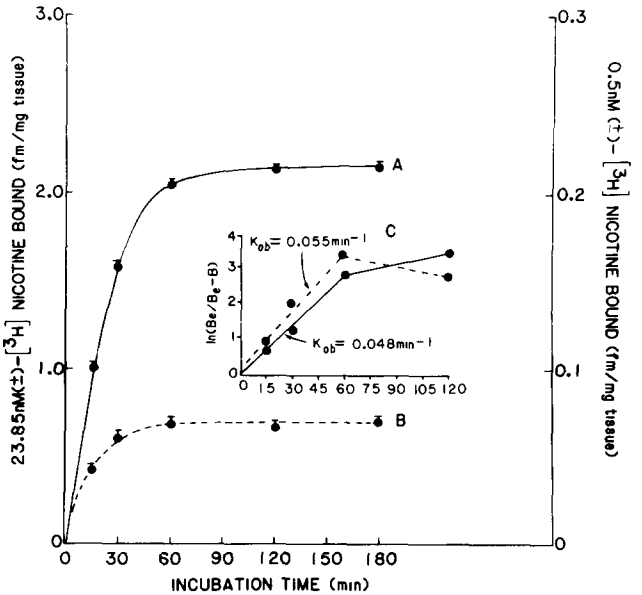

FIG 2 Comparison of the time course of saturable binding of an intermediate [(●—●), 23 85 nM] (Curve A) and a low [(●— — —●), 0 5 nM] (Curve B) concentration of (±)−[³H]nicotine to the rat brain P_2 fraction Each point represents the mean ±S E. of 3 determinations. Inset C shows kinetic plots of the two concentrations of nicotine

Data Analysis

The data were analyzed several ways. An iterative non-linear computerized curve fitting procedure [39] hereafter referred to as LIGAND was employed. The program is available in Basic Language through Vanderbilt University [Biomedical Computing Technology Information Center (BCTIC) R-1302 Vanderbilt Medical Center, Nashville, TN 37232]. As will become apparent subsequently, unequivocal evidence of positive cooperativity has been found with several nicotinic drugs It was empirically observed that variance was large over dose ranges where cooperativity was manifest. It was felt that the weighting of variance might eliminate significant data. For this reason two other analyses of variance were employed to characterize the configuration of Scatchard plots (Table 1). ANOVA I segregated the variances of the bound/free (B/F) values into compartments: (1) The between homogenates and (2) the between concentrations variance. The residual variance was used as an error term. The between concentration variance was further analyzed by determining the variance accounted for by the regression of the B/F values against the appropriate mean B values.

ANOVA II involved iterative fitting of multiple regression lines of B and B/F Scatchard data to achieve the least mean squares. The deviation from regression sum of squares was calculated for each line or set of lines and compared using an ANOVA as illustrated in Table 1 Significance levels (p values) for F ratios were calculated. K_D's were also determined graphically [46]

The statistical significance of the enhanced binding of (±)−[³H]nicotine which occurred in the presence of several concentrations of some drugs was assessed with a two-way ANOVA and t-test.

RESULTS

Characteristics of Nicotine Binding

Figure 1 shows the rate of association and dissociation of (±)−[³H]nicotine to rat brain homogenates. Nicotine binds very rapidly over the first seven or eight minutes and thereafter the rate of binding is slowed (Fig 1A). As can be seen in Fig 1B the dissociation of nicotine appears to also take place in two phases: a very rapid phase followed by a much slower rate of loss of nicotine from the tissue. Figure 2 shows that the kinetics of binding are essentially the same for both a low and intermediate concentration of (±)−[³H]nicotine, a point which will be referred to again subsequently. As can be seen from Figs. 1 and 2, equilibrium was attained after 60 minutes of incubation Figure 3 shows that maximum saturable binding was obtained with a concentration of 40 mg/ml of the P_2 fraction.

Nicotine and Carbachol Binding

Figure 4A shows a Scatchard plot of the saturation bind-

ing curve (4B) obtained using increasing concentrations of (±)−[^{3}H]nicotine. Both the configuration of the curve in 4B and the curvilinear nature of the Scatchard plot in 4A suggest more than one binding site. When these data were fitted by the LIGAND iterative curve fitting program [39] two binding sites were obtained and these are indicated by the dashed lines in Fig. 4A. As can be seen, the open circles, which are the values calculated from these lines [46], are in relatively good agreement with the experimental findings (solid circles). An enhancement of binding was observed at the lowest concentrations (5×10^{-10} M to 2×10^{-9} M; Fig. 4, points 1, 2 and 3) giving the curve a "fishhook" shape. Further, when the data were analyzed using a Hill plot (Fig. 5) the Hill number for the two lowest concentrations was greater than one (1.37)

Figure 6 shows the inhibition curve obtained using a wide range of concentrations of unlabeled (−)−nicotine for the displacement of a single concentration of (±)−[^{3}H]nicotine When all data points were calculated using the LIGAND program a best fit was obtained assuming that the drug had a single K_D, 2.4×10^{-8} M. However, as seen in Figs 6 and 7, some of the determinations made in the presence of 1×10^{-9} M (−)−nicotine resulted in enhanced binding of (±)−[^{3}H]nicotine which also suggested positive cooperativity. In order to obtain some insight into the nature of the binding and to further define the shape of the curve a Scatchard analysis of the displacement data (Fig. 7) was done using ANOVA I and II and the curve was analyzed by determining the lines that best fit it These are illustrated in Fig. 8B As can be seen, data points 7 through 22 (Fig. 7) were best fit by three straight lines (Solid Lines 2, 3 and 4; Fig 8B and C). Of particular importance is the fact that the slope of line 2 (Fig. 8B) was less than the slope of line 3. This could be a consequence of (−)−nicotine producing positive cooperativity over the range of 1×10^{-9} M to 3×10^{-8} M (site 2, Table 2) and we therefore concluded that sites 3 and 4 were maximally cooperated binding sites. Using values 11–22 (Fig. 7) the (−)−nicotine curves were refitted using the LIGAND procedure The resulting best fit indicated 2 binding sites (Table 2, sites 3 and 4).

Figure 6 also shows the inhibition curve produced by carbachol. The configurations of the curves are essentially parallel. Carbachol has an IC_{50} about 2 orders of magnitude greater than nicotine's. It should be noted that the lower concentrations of carbachol (2.5×10^{-9} M to 1.5×10^{-8} M) produced a greater degree of enhanced binding than (−)−nicotine (Fig. 6). The enhancement of binding was significant at $p<0.001$. There was neither a between homogenates nor a between concentrations difference in binding over this range of concentrations Since carbachol is 200 times less potent in inhibiting (±)−[^{3}H]nicotine binding (Fig. 6) concentrations of nicotine (10^{-11} M–10^{-9} M) which were presumed to be equipotent to the cooperating doses of carbachol were studied. The data from these experiments are presented in Figs. 7 and 8 (points 1–6) Figure 8A (solid line 1) presents a Scatchard plot of these data. As can be seen, over this low range of concentrations nicotine did not produce positive cooperativity but instead produced a significant inhibition of binding ($p<10^{-6}$) indicating a very high affinity site (Site 1, Table 2)

The Inhibition of (±)−[^{3}H]Nicotine Binding by (+)−Nicotine

As was the case with (−)−nicotine, the interaction of (+)−nicotine with nicotine binding sites was complex. When the inhibition curve (Fig. 7) was analyzed by the LIGAND program three binding sites were obtained (Table 2, sites 3, 4 and 5 and Fig 8C, Lines 3 and 4). As can be seen from Fig 7 very low concentrations of (+)−nicotine both inhibited and enhanced the binding of (±)−[^{3}H]nicotine; however, neither of these trends are statistically significant. When a Scatchard analysis of the inhibition curve for (+)−nicotine was performed (Fig. 8A), a very high affinity binding site was identified (line 1, points 1 and 2). Points 3 to 6 show negative bound values because of the enhanced binding of (±)−[^{3}H]nicotine As can be seen from Fig. 7 the IC_{50} of (−)−nicotine is one hundred times less than (+)−nicotine's IC_{50}

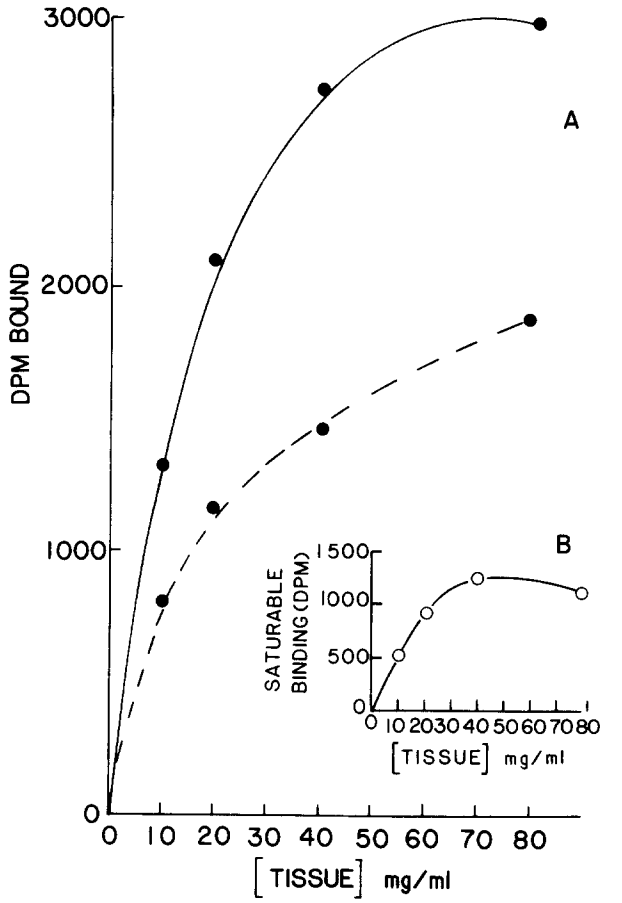

FIG. 3 The relationship of tissue concentration to the amount of saturable binding (±)−[^{3}H]nicotine (23 85 nM) was incubated at 4°C with varying amounts of the P_2 fraction for 60 min Binding in the absence (●—●) and presence (●— — —●) of 10 mM (−)−nicotine is shown in (A) The insert (B) shows the relationship of saturable binding and tissue concentration

Inhibition Curves for Drugs Which Both Enhance and Inhibit the Binding of (±)−[^{3}H]Nicotine

A number of cholinergic drugs besides (−)−nicotine, (+)−nicotine and carbachol produced both an inhibition and an enhancement of (±)−[^{3}H]nicotine binding. These drugs are shown in Fig 9 and Table 2. Lobeline was the most potent of these drugs in inhibiting the binding of nicotine with an IC_{50} of 4×10^{-7} M It also produced a significant

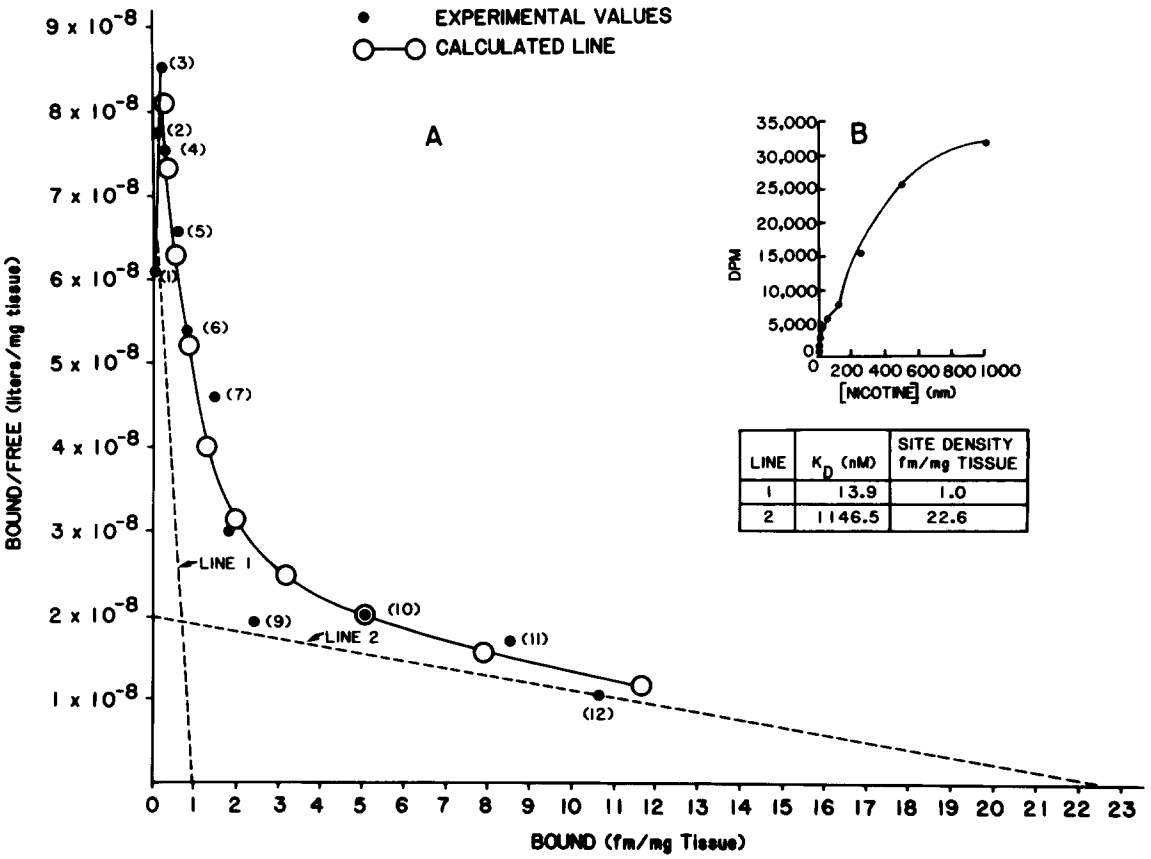

FIG 4. Scatchard plot of (±)−[³H]nicotine saturation binding Concentrations of (±)−[³H]nicotine ranging from 5×10^{-10} M (point 1 in Fig 4A) to 1×10^{-6} M (point 12) were employed Inset (B) shows the saturation curve (corrected for non-saturable binding) A Scatchard conversion of this curve is shown in (A) as solid circles The dashed lines represent the best fit to these data obtained by using the LIGAND program. K_d's and site densities calculated from these lines are shown in the box. The open circles represent the calculated values for the line

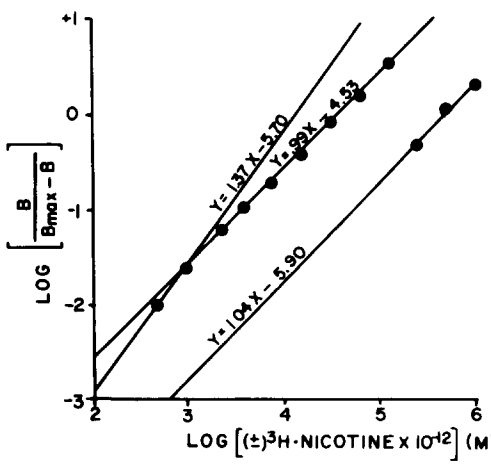

FIG 5 Hill plot of the binding of (±)−[³H]nicotine This plot is derived from the data shown in Fig 4 Points 1 and 2 had a slope of 1 37 whereas points 3 through 9 and 10 through 12 had slopes of 0.99 and 1 04 respectively

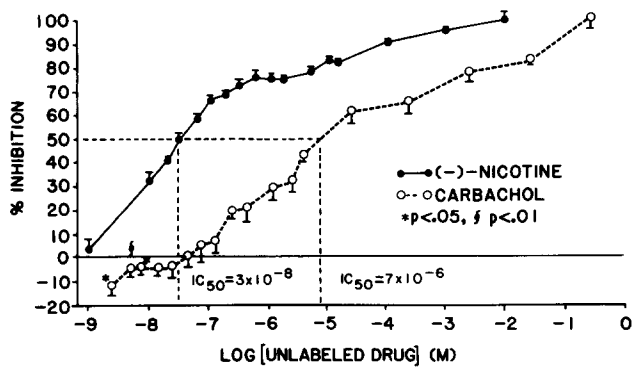

FIG 6 The inhibition of (±)−[³H]nicotine binding by (−)−nicotine and carbachol. The binding of (±)−[³H]nicotine was determined in the presence of graded concentrations of either (−)−nicotine or carbachol The inhibition produced by (−)−nicotine or carbachol is shown as a percentage of the maximum displacement achieved by either 1×10^{-2} M (−)−nicotine or 2.5×10^{-1} M carbachol Each point is the mean of four experiments with its standard error

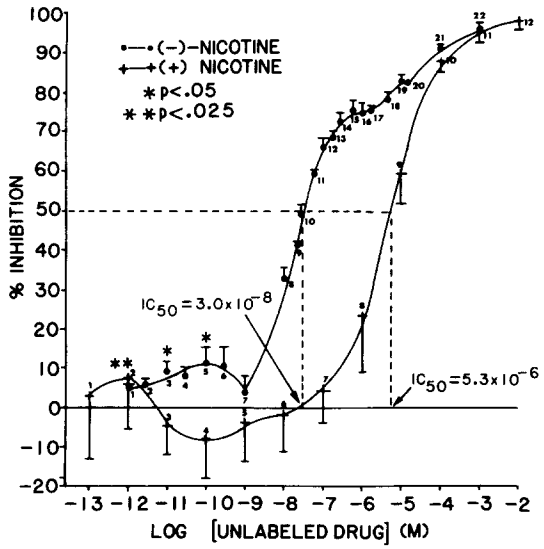

FIG 7 Inhibition of (±)-[^{3}H]nicotine binding by graded concentrations of (-)- or (+)-nicotine The inhibition produced is shown as a percentage of the maximum displacement achieved by 1×10^{-2} M (-)-nicotine Each point is the mean of 4 experiments with its standard error The concentrations of cold ligand are numbered The asterisks indicate the points that are significantly greater than zero using the Student's t-test

enhancement of nicotine binding over a concentration range of 1×10^{-9} to 1.5×10^{-8} M ($p<0$ 05) When the data were analyzed by the LIGAND program one binding site was found (Table 2). Atropine produced a significant enhancement of nicotine binding over a wide concentration range (1×10^{-12} to 1×10^{-5} M, $p<0$ 001) but only a modest inhibition of (±)-[^{3}H]nicotine binding in concentrations up to 1×10^{-2} M. When the data were subjected to the LIGAND program only one binding site was obtained (Table 2). Mecamylamine (Fig. 9 and Table 2) like atropine also enhanced the binding of (±)-[^{3}H]nicotine over a wide concentration range and produced little inhibition of binding even at 2.5×10^{-2} M (<20%) A statistically significant amount of enhanced binding was seen at two concentrations, 5×10^{-9} M and 1.5×10^{-8} M. Further there was a significant difference between homogenates variance in concentration ranging from 2.5×10^{-9} M to 2.5×10^{-4} M, $F(3,42)=2$ 83, $p<0.05$, but no difference between concentrations The enhancement of nicotine binding was significant ($p<0.025$). Acetylcholine produced a greater degree of enhanced binding than any drug studied (Fig. 9) and was the least potent inhibitor of (±)-[^{3}H]nicotine binding. In the concentration range of 2×10^{-8} to 3×10^{-7} M where an analysis of variance indicated that there was no significant between dose variance the pooled data showed a significant enhancement of binding ($p<0$ 001) In contrast, higher concentrations ($5\ 4\times10^{-7}$ M to 1×10^{-3} M) produced increasingly less enhancement of binding When the curve was fitted by LIGAND, three binding sites (sites 2, 3, 4, Table 2) were obtained The positive cooperativity site may represent a high affinity site distinct from fully cooperated low affinity sites for acetylcholine using these procedures

Drugs that Inhibit (±)-[^{3}H]Nicotine Binding

Cytisine, anabasine, choline and cotinine inhibited (±)-[^{3}H]nicotine binding without producing positive cooperativity Concentrations [based on potency ratios of the IC_{50}'s relative to (-)-nicotine] were used that were less than or about equipotent to the lowest doses of (-)-nicotine (Fig 10, Tables 2 and 3). Cytisine is the most potent of any drug reported in this study excepting (-)-nicotine in its ability to inhibit nicotine binding (Fig. 9, Tables 2 and 3) The LIGAND curve fitting programs indicated that a single line was the best fig for the data. The anabasine curve has an IC_{50} about 480 times greater than cytisine's (Fig 10, Table 3) and about 260 times greater than (-)-nicotine (Fig. 9, Tables 2 and 3) A best fit of the data was obtained assuming two binding sites (Table 3) Both choline and cotinine had a very low affinity for nicotine binding sites. A single line provided the best fit of the choline data The cotinine data was best fit with two lines (Table 3)

DISCUSSION

Under the conditions of this assay (±)-[^{3}H]nicotine binds to multiple sites in the rat brain P_2 preparation by complex but saturable, reversible and stereospecific processes The binding and dissociation of nicotine has a very rapid and a slow component Both processes attain equilibrium by 60 minutes and remain stable for at least 120 minutes Table 4 summarizes the IC_{50}'s of different ligands obtained by other investigators As can be seen there is excellent agreement between laboratories for all ligands excepting cotinine and acetylcholine Difference in buffers probably accounts for these exceptions. The binding affinity of acetylcholine has been shown to be markedly affected by buffer concentration and by the buffer [55] Studies with cotinine were conducted using Tris buffer [3] Tris and 50 mM Hepes confers different binding characteristics to some ligands

The presence of both high and low affinity nicotine binding sites is also in agreement with previous reports [1, 14, 43, 54, 56, 59, 60] However, in the present studies several lines of evidence show that (-)- and (+)-nicotine as well as other ligands produce positive cooperativity The "fishhook" shape of the Scatchard plot at concentrations ranging from 5×10^{-10} to 2×10^{-9} M together with a Hill coefficient of 1 37 for this segment of the curve are consistent with a high affinity positive cooperativity site This positive cooperativity at low ligand concentrations does not appear to be a consequence of a failure to attain equilibrium since saturable binding at both low (5×10^{-10} M) and intermediate ($2\ 4\times10^{-8}$ M) concentrations of (±)-[^{3}H]nicotine proceeded at the same rate and achieved equilibrium at 60 minutes Two types of inhibition studies also suggest that (-)- and (+)-nicotine produce positive cooperativity (1) Not only did low concentration of (-)-nicotine increase the binding of (±)-[^{3}H]nicotine in some homogenates, the multiphasic nature of the Scatchard plot was interpreted, in part, as indicating that (-)-nicotine was cooperating the high affinity binding site over a wide range of concentrations (see results for the argument) A similar case can be made for (+)-nicotine which enhanced (±)-[^{3}H]nicotine binding over a lower range of concentrations (2) Ligands differed markedly in their ability to produce positive cooperativity. Thus ACh produced a high degree of cooperativity while cytisine, anabasine, cotinine and choline were devoid of this action

Physiologic and neurochemical studies indicate that the acetylcholine receptor can be cooperated It has been shown that carbachol in low but not high concentrations enhances

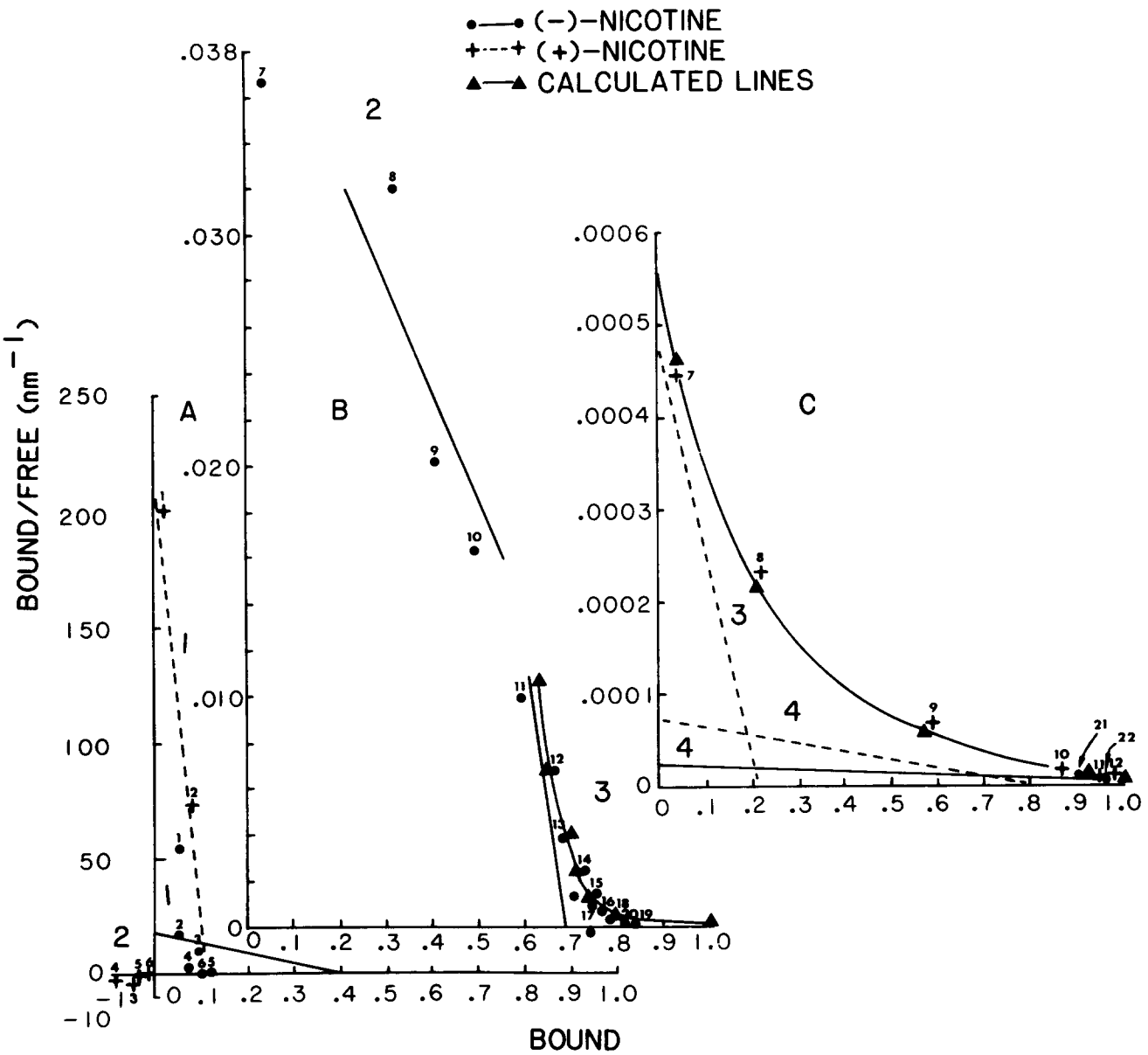

FIG 8 Scatchard analysis of the inhibition of (±)−[^{3}H]nicotine binding by graded concentrations of (−)− and (+)−nicotine Bound (shown on the abscissa of panels A, B and C) represents the combined fraction of (±)−[^{3}H]nicotine and cold (−)−nicotine that is saturably bound at each point. Bound/free (the ordinate for each graph) is derived by dividing the fraction bound by the concentration of the free drug The large numbers refer to line segments (binding sites and K_d estimates, Table 2) whereas the small numbers refer to cold ligand concentrations designated in Fig 7 Panel A shows Scatchard conversions of points 1–6 for (−)−nicotine and (+)−nicotine The solid line 1 refers to a very high affinity site for (−)−nicotine (Site 1, Table 2) whose K_d was generated by the best fit line to data points 1–6 using ANOVA I and ANOVA II The dashed line is for a very high affinity site for (+)−nicotine whose $K_d$was calculated from the slope of the line between data points 1 and 2 (Site 1, Table 2), whereas the large 2 over points 3 to 6 designate the concentrations which produce positive cooperativity (Site 2, Table 2). Panel B encompasses (−)−nicotine data points 7–20 The large 2 refers to data points 7–10 (Site 2, Table 2) The line through points 8–10 represents the best fit line obtained by ANOVA I and ANOVA II Data points 11–22 were fitted by the LIGAND program The slopes of the lines calculated from the K_d's are line 3, Panel B and line 4, Panel C. Panel C for (+)−nicotine also shows data points 7–12 which were fitted with the LIGAND program These data were best fitted by lines 3 and 4 (Sites 3 and 4, Table 2) yielding the indicated calculated line Site 5 for (+)−nicotine shown on Table 2 has bound/free values too low to show graphically in Panel C

TABLE 2

INHIBITION OF NICOTINE BINDING BY DRUGS THAT PRODUCE POSITIVE COOPERATIVITY

Drug	Binding Site	Positive Cooperativity Range (M)	K_D (M)	IC_{50} (M)
(−)−Nicotine	1		2.2×10^{-11}	
	2	1×10^{-9} to 3×10^{-8}		3×10^{-8}
	3		5.2×10^{-9}	
	4		4.5×10^{-5}	
(−)−Lobeline	1	1×10^{-9} to 1.5×10^{-8}		
	2		2.8×10^{-7}	4×10^{-7}
(+)−Nicotine	1		4.9×10^{-11}	
	2	1×10^{-12} to 1×10^{-9}		
	3		4.3×10^{-7}	5.3×10^{-6}
	4		1.1×10^{-5}	
	5		4.3×10^{-2}	
Carbachol	1	2.5×10^{-9} to 2.5×10^{-7}		
	2		5.3×10^{-6}	7.0×10^{-6}
	3		4.7×10^{-2}	
Mecamylamine	1	2.5×10^{-9} to 2.5×10^{-4}		
	2		5.8×10^{-3}	$>1\times10^{-3}$
Atropine	1	1.0×10^{-12} to 1×10^{-5}		
	2		3.6×10^{-4}	$>1\times10^{-3}$
Acetylcholine	1	2×10^{-9} to 3×10^{-7}		
	2		1.7×10^{-6}	
	3		2.9×10^{-5}	2.5×10^{-2}
	4		3.6×10^{-2}	

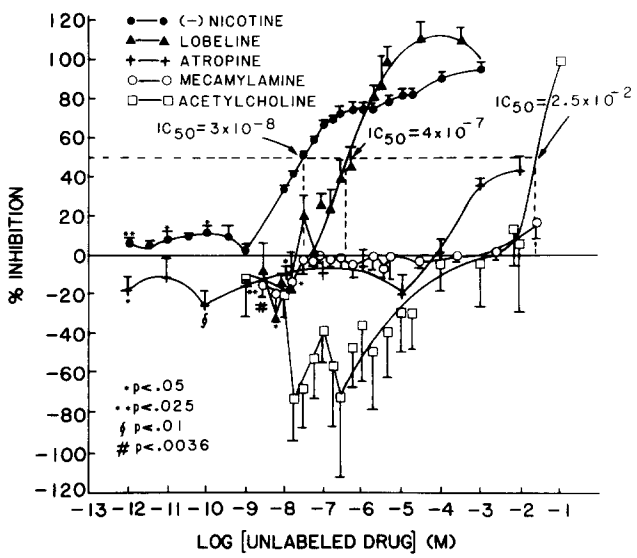

FIG 9 Drugs that significantly enhanced and inhibited nicotine binding: lobeline, atropine, mecamylamine and acetylcholine The inhibition produced by each drug is shown as a percentage of the maximum displacement produced by 1×10^{-2} M (−)−nicotine except lobeline (compared to 1.25×10^{-3} M lobeline) and acetylcholine (compared to 1×10^{-1} M acetylcholine) Each point, shown with its standard error is the mean of four experiments except for lobeline (3 experiments) and atropine (8 experiments) Symbols indicate the level of significance of the values

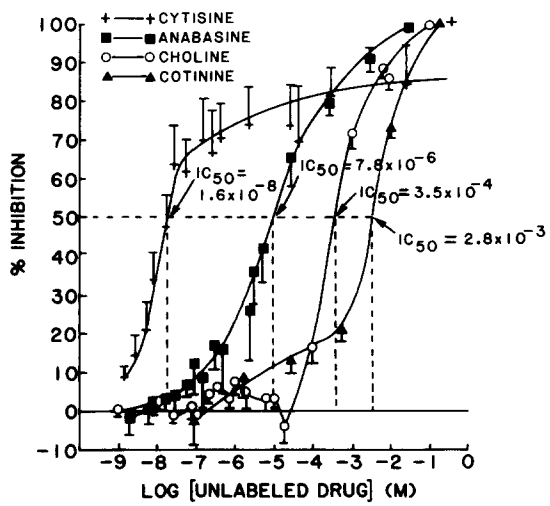

FIG 10 Drugs that produced no statistically significant enhancement of nicotine binding cytisine, anabasine, choline and cotinine Each point shown with its standard error, is the mean of four experiments except for cotinine which is the mean of three experiments.

TABLE 3

INHIBITION OF NICOTINE BINDING BY DRUGS WHICH DO NOT PRODUCE POSITIVE COOPERATIVITY

Drug	Binding Site	K_D (M)*	IC_{50} (M)
Cytisine	1	5.0×10^{-9}	1.6×10^{-8}
Anabasine	1	2.6×10^{-6}	7.8×10^{-6}
	2	1.3×10^{-3}	
Cotinine	1	6.2×10^{-7}	
	2	4.7×10^{-3}	2.8×10^{-3}
Choline	1	4.8×10^{-4}	3.5×10^{-4}

*K_D's calculated from the data shown in Fig. 10 using LIGAND.

fluorescence due to the covalent binding of 5-(iodoacetamido) salicylic acid (a probe known to alkylate the reduced nicotinic acetylcholine receptors) to the Torpedo nicotinic receptors [19]. It has further been shown that carbachol enhances the binding of [^{3}H]phencyclidine to the channel site of the acetylcholine receptor of the Torpedo [20]. Carbachol, nicotine and acetylcholine markedly increased the rate and equilibrium levels of [^{3}H]perhydrohistrionicotoxin binding to Torpedo membrane nicotinic receptors [8]. Nicotine, in doses which did not contract the nictitating membrane of the chloralose anesthetized cat potentiated submaximal responses to low rates of preganglionic stimulation [7]. The fact that drugs which are presumed to occupy the high affinity nicotine binding site differ markedly in their ability to produce positive cooperativity argues that it is a separate site. The physiologic and pharmacologic significance of this site is not known.

The very high affinity binding site observed in the present study is in keeping with the very high affinity site recently reported ($K_D=2\times10^{-10}$ M) where (−)−[^{3}H]nicotine was used as the radiolabeled ligand [1]. Although (+)−nicotine was almost as effective as (−)−nicotine in displacing (−)−[^{3}H]nicotine from this site these investigators revealed in a personal communication that the very high affinity site was not seen when (+)−[^{3}H]nicotine was used as the radioligand. This apparently paradoxical observation can be explained by assuming that (−)−[^{3}H]nicotine also alters the very high affinity site, increasing its affinity for (+)−nicotine.

TABLE 4

THE IC_{50}* VALUES OF A VARIETY OF LIGANDS FOR INHIBITING (±)-[^{3}H]NICOTINE BINDING TO RAT BRAIN TISSUE THE PRESENT STUDY COMPARED WITH THE LITERATURE

	IC_{50} M		
Ligand	Present Study	Other Investigators	Reference
Cytisine	1.6×10^{-8}	1.4×10^{-8}	43
(−)−Nicotine	3.0×10^{-8}	6.2×10^{-8}	43
		2.6×10^{-8}	3
		6.0×10^{-8}	14
(+)−Nicotine	5.3×10^{-6}	3.9×10^{-6}	43
		13.0×10^{-6}	3
		3.3×10^{-6}	4
Lobeline	4.0×10^{-7}	3×10^{-7}	43
		1.2×10^{-7}	14
Anabasine	7.8×10^{-6}	4.6×10^{-6}	43
		22.0×10^{-6}	3
Carbachol	7.0×10^{-6}	2.4×10^{-6}	43
		4.3×10^{-6}	3
		0.9×10^{-6}	14
Choline	3.5×10^{-4}	3.4×10^{-4}	14
Cotinine	2.8×10^{-3}	4.3×10^{-6}	3
Acetylcholine	2.5×10^{-2}	2.8×10^{-7}	14
Mecamylamine	$>1.0\times10^{-3}$	$>1\times10^{-3}$	43
		2.5×10^{-4}	14
Atropine	$>1.0\times10^{-3}$	$\sim1.0\times10^{-3}$	43
		8.8×10^{-4}	14

*The concentration necessary to inhibit the saturable binding of (±)−[^{3}H]-nicotine by 50%

In the present study stereospecificity of nicotine binding was demonstrated for some but not all sites. Although similar K_D ratios for (+)/(−)−nicotine were observed for both the very high affinity site (Site 1) and the low affinity site (Site 4), (+)−nicotine was more effective in enhancing the binding of (±)−[^{3}H]nicotine (Site 2). In contrast, (−)−nicotine was more effective (80 times) than (+)−nicotine in inhibiting binding at the high affinity site (Site 3).

TABLE 5

POTENCY RATIOS OF (+)/(−)−NICOTINE IN PRODUCING DIFFERENT PHARMACOLOGIC EFFECTS IN THE RAT

Effect	Potency Ratio	Route of Administration	Reference
Lethality	1	IP	22,28
Discriminative Stimulus	9	SC	33
Analgesia	Ca 30	SC	5
Vasopressor Response	8–17	IP, IA	6, 9, 23
Inhibition of Conditioned Avoidance	7	SC	16
Convulsant Action	6	IV	16
Myoneural Transmission	1	Bath	9,23
Prostration	45–60	IVC	3,4

The correlation of binding specificity with pharmacologic effects is complicated. The relative potency of the (+) and (−) isomers in the rat depends upon the effect studied (Table 5) (−)−Nicotine is 30–60 times more potent than (+)−nicotine in producing prostration and analgesia. This potency ratio of (+)/(−)−nicotine roughly correlates with the ratio observed in binding studies for the high affinity site (Site 3). On the other hand, (−)−nicotine is only 6–17 times more potent than (+)−nicotine in inhibiting conditioned avoidance, in producing convulsions, as a discriminative stimulus and in altering vasomotor activity. The two isomers are equipotent in causing death and in inhibiting transmission at the myoneural junction. They may be nearly equal in their ability to inhibit binding at the very high and low affinity sites.

Brain levels of nicotine following pharmacologically effective doses range from about 1×10^{-9} M to 1×10^{-8} M [29, 44, 45]. These concentrations are consistent with the assumption that some of the central actions of nicotine may be mediated by the high affinity binding site (Site 3) whose K_D is 5.2×10^{-9} M.

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